CYTOLOGICAL STUDIES OF MARSUPIAL AND MONOTREME CELLS IN TISSUE CULTURE

by

Yadviga Alexandra Elizabeth Bick

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Y. a. E. Bick

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A. General Introduction

Tissue culture of animal cells can be said to have begun as early as 1885 when Roux[1] successfully maintained an explant of the medullary plate of a chick embryo. Improvements in culture techniques followed rapidly and Burrows[1], working in the 1920's with the famous French scientist, Alexis Carrel, found that embryo extract had a strong growth promoting effect on certain cells. Carrel[1], already a Nobel prize winner for his work in experimental surgery, was largely responsible for developments in method in the next few years. Bringing with him a knowledge of aseptic techniques, he tackled tissue culture as he would a surgical operation. He thus obtained consistently successful results and performed the staggering feat of keeping a strain of cells in active multiplication for 34 years, and that at a time when there were no antibiotics and when methods were painstaking and tedious. Carrel's work suggested that cells might be grown almost indefinitely like protozoa or micro-organisms, and by the use of large numbers of cells opened up possibilities for the study of cell metabolism.

Baker and Carrel[1] became interested in the composition of the medium required for cell growth in vitro and attempted by analytical procedures to identify the essential constituents in it. Their work was extended by Warren and Margaret Lewis[2]. In addition, the perfection of our present methods of culture owes a great deal to the National

Cancer Institute of U.S.A. headed by Dr. Witton Earle[1] whose research team was the first to grow cultures from single cells or clones. At present, numerous media for tissue culture are commercially available and the work of maintaining cultures has been made relatively easy.

The main improvements in technique that up till now have given the greatest impetus to tissue culture are firstly, the introduction of antibiotics such as penicillin and streptomycin, and secondly, the use of chelating agents such as trypsin and versene (ethylene diamine tetraacetic acid or EDTA) for the disaggregation of animal tissues prior to culturing. Previous to this, small sections of organs were placed in suitable media with the hope that cells would migrate out of the explant and establish themselves in sufficient numbers. Dr. Honor Fell[1], working in the Strangeways Laboratory in Cambridge, made good progress in maintaining in vitro small fragments of organs in which the normal tissue architecture was preserved[1]. Such culture conditions maintained the differentiated state of the cells, for usually cultivation in vitro promotes the morphological and biochemical simplification of the cell population. As a result of this, in a very short time all cultured cells revert to one of three types of cells, often irrespective of their origin[3]:

(1) epithelial cells which tend to grow in sheets with their sides closely attached to other cells like paving stones (HeLa cells, many kidney cells from various mammals)

(2) fibroblast cells, spindle shaped, much longer than they are

broad, which extend radially into the medium to form a network of cells (most testis cultures), and

(3) wandering cells like the leucocytes of peripheral blood which settle at the bottom of the vessel. They move by extending pseudopodia, and even when adhering to the surface are easily detached by a slight shaking of the vessel.

It is possible that differentiation and growth by rapid cell multiplication are mutually exclusive. A mesenchyme cell or fibroblast from periosteal tissue of a mammal will not under the usual culture conditions form bone, although it may not have lost its capacity for doing so. Under culture conditions, it remains undifferentiated. Moscana[4,5] (1961) thought that dedifferentiation of cells in cell cultures was partly due to contact of cells with the floor medium interface, and cell behaviour in such cultures instead of reflecting first order reactions was frequently conditioned by the surface structure of the floor medium interzone. To prevent this he used rotating flasks; in this way submitting the cells to continuous effect of controlled centripetal forces of low intensity. This eliminates almost completely prolonged cell-floor contact, while considerably expediting the compounding of cells into aggregates. By this method he obtained quite a marked differentiation of cells in his cultures.

The expectation that the reduction of tissues to their component cells could lead to a better understanding of their specific metabolism has not always been fulfilled, mainly due to the cellular dedifferent-

iation which has limited the usefulness of the cell culture technique. However, tissue culture has helped in the better understanding of certain physiological problems. Cavanaugh[6] and later Harary and Farley[7,8,9] have shown that some of the embryonic chick heart cells grown in culture show rhythmical contractions. About 2% of these cells once attached to a glass surface begin to beat - a majority at 60 beats per minute, a few as low as 10 and others as high as 180 beats per minute. Once the cells join by processes to form a close network, they lose their independence and beat synchronously. The rate of beating for the synchronous net is determined by the fastest cells. Despite the superimposed rate, each cell retains potentially its own inherent rate of beating. These experiments indicate that organization and function of adult tissue is inherent in the component cell and that the organization of the heart also, in large measure, depends on the ability of single cells to communicate. It also shows that the adult mammalian heart activity is not determined by neural activity, but that neural regulation is superimposed. To quote Carrel - "The organism is one cell that has divided and become a multitude at the same time as it has retained its unity."[10]

As with any new tool or new experimental method, tissue culture has opened up new horizons in biology. In virology, its widespread application was initiated by the observations of Enders and his associates[1] in 1949 that poliomyelitis virus would grow in human tissues of non-nervous origin. It has now been shown that many human viruses will grow readily

in practically any cells of human and monkey origin.

In recent years (1960), the discovery by Nowell[11] that phytohaemagglutinin (PHA) can initiate mitoses in peripheral leucocytes under culture conditions has led to further developments in cytology. PHA, a protein with a mucopolysaccharide prosthetic group, is obtained as a crude extract from the common bean (Phaseolus vulgaris) and was originally used as a means of separating the plasma from the erythrocytes which became agglutinated by PHA[12]. The blood leucocytes having a lower specific gravity than the erythrocytes are left in the supernatant plasma, and Nowell discovered that such leucocytes after a few days at 37°C exhibit mitoses. Normally, the peripheral leucocytes of blood are highly specialized cells which have lost all power of dividing in healthy individuals, so that when they are stimulated artificially to divide, we have a synchronous population of dividing cells all of which are in G1 or pre-DNA synthetic phase before the addition of PHA. This facilitates immensely experiments in irradiation, and one can be quite certain of the stage of the cell cycle and perfect synchronization of the cells at the time the Xray dose is delivered. Cultures of peripheral leucocytes have enabled the karyotype of individuals to be obtained readily and chromosomal abnormalities can be determined quickly. Microtechniques can yield a chromosome count from an individual in a few days from as little as a few drops of blood from a finger tip. Karyotypes of unstudied species, the sequence of DNA replication, the relative DNA content of various animals, studies of sex-mechanism by the combination

of autoradiography with tissue culture techniques are all within easy reach. In the future, progress in tissue culture may well lead to solving the problems of differentiation and specialized cell function at a level below that of the whole organism.

The work reported in this thesis was begun with the study of the effect of the alkaloid, heliotrine, which mimics the effects of Xrays, on mitotic inhibition and chromosomal breakage in marsupial leucocytes *in vitro*. Heliotrine occurs naturally in Australian pasture plants and causes severe liver damage in sheep[13] that have grazed for two consecutive seasons on plants containing it. Although some work had been done on the damage produced by heliotrine in plant chromosomes[14] and it had been shown to be a powerful mutagen in the fruit fly, *Drosophila*[15], nothing was known about its effect on mammalian chromosomes. The Tasmanian rat-kangaroo, *Potorous tridactylus*, was deemed a suitable experimental animal: its low chromosome number and distinct chromosomal morphology facilitate metaphase scoring of damage. Even a single chromosome from a disrupted cell nucleus can be identified with confidence and certainty. Leucocyte cultures were most suitable for this study and a few millilitres of blood were sufficient for a number of experiments. The blood could be obtained from the tail of the animal which meant that an animal could be used for a number of experiments without being sacrificed.

Heliotrine belongs to the pyrrolizidine group of alkaloids, many of which are potent mitotic poisons and have a selective hepatotoxic

action[16,17]. Chemically the same alkaloids are powerful alkylating agents and their toxicity may be associated with this[19]. They are present in the composite Senecio spp. which are used for herbal remedies in South Africa and Java. This is reflected in the number of deaths due to cancer of the liver: in Europe the percentage of deaths due to this cause is 1.2%, whereas the toll for the Javanese and Bantu is 41.6 and 50.9%[18]. The study of chromosomal damage by heliotrine may throw some light on the action of carcinogens that are powerful alkylating agents.

A comparison of the effect of heliotrine with Xray damage led to the widening of this study to include the problems of chemical protectors against radiation and that of chromosomal repair in relation to the cell cycle by the use of protein inhibitors. This work too has been carried out on the leucocytes of Potorous in vitro. These experiments were interrupted for several months due to failure of the marsupial leucocytes to divide; human leucocytes on the other hand under exactly similar conditions flourished and divided. The trouble was finally traced when it was found that the serum had become toxic or at least mitosis-inhibiting at the levels used. The problem was solved by lowering the serum concentration from the former 20% to 8%.

Long term cultures from potoroo kidney and testis material had been established successfully by this time, and during the lull in the work involving leucocytes, experiments were undertaken on the permanent marsupial cell lines. The potoroo kidney epithelial line is especially

ideal for laboratory work: it has a generation time under 24 hours; after nearly 4 years in the laboratory it is still 90% diploid and the cells have retained their contact inhibition. There is virtually no aneuploidy and the morphology of the chromosomes does not seem to have altered at all since the initiation of the primary cultures. Unfortunately, work with permanent cultures is extremely time-consuming and exacting, and because of the quick generation time of the cell line, subculturing has to be done every four to five days. Long-term cultures, however, provide good training in aseptic technique and the potoroo kidney cell line has been distributed to other laboratories, and batches of cells have been sent to Canberra, Sydney, Adelaide, Fort Chase, U.S.A. and to Rome.

By this time some degree of experience with the new techniques had been acquired and it seemed an opportune moment to study chromosome numbers of the platypus and echidna which had not previously been determined. In Tasmania, the two monotremes are plentiful and easily trapped: the female platypus which was used in the experiments was actually found wandering in a busy street a mile from the centre of Hobart! Recently, the work of Ohno[20,21,22] and his coworkers on the relationship of the placental mammals to other vertebrates, in respect to the relative DNA cell content, has aroused much interest. Experiments, based on Ohno's technique of using chromosomal areas from cells dividing in culture, were carried out to determine the status of the echidna and platypus in this regard.

The results of these studies are reported in separate sections of the thesis.

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B. General Summary

Section A. General Introduction.

Section C. The Karyotype and possible Sex-mechanism of the Monotremes *Ornithorhynchus anatinus* (Platypus) and *Tachyglossus aculeatus* (Echidna).

Conditions for culturing leucocytes of the monotremes and the preparation of testis and spleen tissue for chromosomal preparations of these animals is given in detail.

The chromosome numbers and morphology of the male and female of the species are described, and the possible sex-mechanism is discussed. The monotreme karyotype is compared with that of other vertebrates.

Section D. The DNA Content of the Monotremes in Relation to other Mammals by Biochemical and Cytological Estimations.

The DNA content of fresh heart tissue from the two monotremes (platypus and echidna), the marsupial potoroo, and the rat (as a representative of a placental mammal) was found by biochemical estimations. The genetic content of echidna, man and potoroo was determined from chromosomal area estimations of colchicinized leucocytes in tissue culture. The $\frac{X}{A + X}$ ratio for the echidna proved similar to that of placental mammals. The relationship of the monotremes to other vertebrates in the light of these results is discussed.

Section E. Effects of Heliotrine on the Mitotic Index and Chromosomal Breakage in Marsupial Cells *in vitro*.

The effects of the pyrrolizidine alkaloid heliotrine on the inhibition

of dividing leucocytes of Potorous tridactylus was investigated. Types of chromosomal aberrations were found for different levels of heliotrine concentration. The presence of chromosomal breaks as well as chromatid breaks after heliotrine treatments indicates that the action of heliotrine in effecting chromosomal damage is not restricted to the replication phase of the cell cycle as is usual for other alkylating agents. A statistical comparison was made of the distribution of breaks after X-irradiation and heliotrine treatments.

Section F. Chemical Protection against X-irradiation by a new reducing Agent, 1,4-Dithiothreitol (DTT) in marsupial Leucocytes in Culture.

Leucocytes of the marsupial potoroo were irradiated in the presence and in the absence of DTT, and subsequently cultured for 72 hours. In its presence, the number of aberrations was reduced to half that found in its absence. The action of sulfhydryl compounds as radioprotectors is discussed with special reference to BAL and DTT.

Section G. Modification by Chloramphenicol (CAP) of Radiation-induced Chromosomal Damage in Cultures of marsupial Leucocytes.

CAP was added for varying periods of time to irradiated leucocytes of the marsupial potoroo. The chromosomal aberrations in the nuclei in mitosis after 72 hours of culture showed that the presence of CAP, especially during the final 24 hours of the culture period, increased the number of resultant breaks. These results indicate that some repair (involving protein synthesis) takes place during this part of the culture period, i.e. at least 48 hours after the Xray dose has been delivered.

Section H. Long-term Cultures of marsupial Cells.

Techniques for tissue culture and the setting up of primary and permanent cell lines are given in detail. Experiments on cold treatments of a permanent cell line were carried out. Hybridization was tried between a marsupial cell line and HeLa (human carcinoma) cells. A stable marsupial line (HPK1) of epithelial kidney cells from the male potoroo was obtained which, after four years in vitro, still exhibited 90% diploidy and has kept its original contact inhibition. Irradiation experiments (with dose fractionation) were performed on the HPK1 cells.

C. The Karyotype and possible Sex-determining mechanism of the Monotremes *Ornithorhynchus anatinus* (Platypus) and *Tachyglossus aculeatus* (Echidna)

Introduction

Recent advances in cytological techniques have made it possible to determine chromosome numbers of animals whose karyotype had not been known with certainty because of large chromosome numbers and small size of some of the chromosomal complement. The discovery by Nowell[1] that phytohaemagglutinin (a protein with a mucopolysaccharide prosthetic group, obtained readily as a crude extract from the common bean) can initiate mitotic division in blood leucocytes cultured *in vitro* has made it possible to determine the karyotype of an animal from as little as a few drops of blood. Hypotonic treatment produces excellent spreads of chromosomes without the use of squash techniques, and the addition of colchicine for the final two hours of the culture period increases the number of cells arrested at metaphase and further facilitates the determination of chromosome numbers and morphology.

In placental mammals, chromosome numbers vary over a wide range (from 18 to 86 for the diploid number), although the DNA content has been found to be relatively constant for all species. Marsupials are characterized by large chromosomes and small numbers (2n = 10 to 22), but the DNA content and volume accord with those of eutherian mammals.

Little information has been recorded about the chromosomes of the monotremes. The karyotype of the platypus has been investigated by

Matthey[2] (1949) although with the techniques available at that time the chromosome number (70 ± 10) could not be ascertained with accuracy. Matthey remarked that the karyotype in the monotremes shows a division into macro and micro elements and is similar to that in birds and reptiles. Van Brink[3] (1959) published a report on the karyotype in Echidna giving numbers for the male between 62 and 64. She recorded one large chromosome as being unpaired, but the scarcity of divisions in the material available did not allow for a decision between an XO and XY heterogamety.

In most mammalian species studied there is a typical XX-XY sex chromosome mechanism, and the male is the heterogametic sex. However, there is an XX-XY_1Y_2 mechanism in two macropod marsupials, Potorous tridactylus[4,5] and Wallabia bicolor[6], and at least one eutherian mammal, Sorex araneus[7]. A more complex sex-determining system, $X_1X_1X_2X_2$ in the female and X_1X_2Y in the male, has been found in two placental mammals[8,9] and recently in an Australian marsupial, the hare-wallaby[10]. The sex-determining system in the Monotremata is completely unknown.

In this work an attempt has been made to resolve the sex-determining system of the monotremes and to determine their karyotype. Metaphases from colchicine-treated short-term cultures of leucocytes from the peripheral blood of the male and female echidna and platypus gave suitable preparations from which the chromosome number could be determined without ambiguity. In addition, chromosome spreads from two male echidnas

were obtained by subjecting testis material to trypsinization and hypotonic treatment for examination of mitosis and meiosis in spermatogenesis. The testes of the male platypus available for this study were small and undeveloped and no divisions were found. Good chromosome preparations were obtained, however, from the spleen of the female platypus.

Both the monotremes are highly protected species. Although the platypus and echidna are much more common in Tasmania than in other Australian states, the platypus is rarely seen, and presents a further problem since it often dies of shock after a few hours in captivity. The breeding season, about which little information is available, is of short duration; and the scarcity of divisions in the gonads, which often regress for considerable periods, make the study of testicular meiosis extremely difficult without sacrificing large numbers of animals.

Materials and Methods

Chromosome spreads from testis

The testes of a mature echidna were removed, chopped finely, and then subjected to trypsinization (0.25% trypsin solution) with agitation by a magnetic stirrer for 20 minutes. The supernatant was removed and the cells were resuspended in balanced saline solution; clumps were broken up by gentle pipetting. This cell suspension was then transferred to fresh tubes (1/2 ml in each) and 1½ ml of warm (37°C) distilled water was added to each tube. The contents of the tubes were thoroughly pip-

etted to ensure penetration of the hypotonic solution and to prevent any clumps of cells forming, and were then immersed in a water bath at 37°C for 12 minutes. After this time, glacial acetic acid and absolute ethanol fixative (1½ ml of 1:3) was added to each tube, and the cell suspension was again gently pipetted. The tubes were allowed to stand at room temperature for half an hour. They were then centrifuged, the supernatant was removed and replaced by 1½ ml of freshly prepared fixative. After 30 minutes, slides were made by pipetting a couple of drops onto a clean slide and adding 4 to 5 drops of 50% acetic acid. The slides were dried either by gentle heating over a bunsen flame or on a warm plate (50°C). Slides were rinsed in absolute methanol, air dried, and stained for 10 minutes with Leishman's stain.

Chromosome Preparations from Spleen

A female platypus was injected with 1 ml of 0.1 per cent colcemid dissolved in sterile balanced salt solution. After 2 hours, the animal received an injection of Nembutal (1 ml per 5 lbs body weight). The spleen was removed, cut up finely and treated for 15 minutes with trypsin (0.25 per cent) with mechanical stirring at room temperature. The cell suspension was washed in balanced saline, and after hypotonic treatment for 12 minutes it was fixed and slides were made and stained as for testis cells.

Leucocyte Culture

The leucocytes were cultured by a modified method of Moorhead et al.[11]. Medium 199 (Parkers) with 10% foetal calf serum was used

(both obtained from the Australian Commonwealth Serum Laboratories). The phytohaemagglutinin (PHA, Wellcome) was dissolved in complete medium. The final concentration of PHA for echidna leucocytes was 5½% (1 mg/ml) and 2% and 5½% for platypus leucocytes.

Blood was removed from the heart with a heparinized sterile syringe and 5 ml aliquots were placed in tubes containing a couple of drops of heparin and PHA. After 10 minutes the blood was gently centrifuged at 100g for 5 minutes until about 40% of leucocyte suspension in plasma appeared on top. This upper layer was transferred to fresh tubes and centrifuged for 10 minutes at 150g when all the leucocytes formed a pellet at the bottom. All the supernatant was removed and the cells resuspended in 4 ml of complete medium. After centrifuging for 10 minutes, the supernatant was again removed and 1 ml of complete medium was added to each tube. Half of this suspension was transferred to each culture bottle which contained 8½ ml of complete medium.

Echidna cultures were incubated at 27, 33 and 37°C with colcemid (Ciba) added for 2, 48 or 58 hours of the final culture period. The final colcemid concentration was either 0.007% or 0.01%. Cultures were stopped on the 3rd, 4th, 5th, 6th, 7th, 8th and 9th day.

Platypus cultures were incubated at temperatures of 27, 31, 35 and 37°C with colcemid (0.01%) added for 4, 20 and 40 hours of the final culture period. Cultures were stopped on the 3rd, 4th, 7th, 8th and 9th day.

Unlike mammalian or marsupial leucocytes, those of the echidna and platypus tend to adhere very firmly to the bottom of the glass vessels, and in some cases versene had to be used to free the cells. The leucocytes remaining in suspension tend to form clumps, so that vigorous pipetting is essential before hypotonic treatment can be applied. After the leucocytes were separated from the culture medium by centrifuging, the supernatant was removed except for ½ ml in which the cells were resuspended. The cells were then given hypotonic treatment, fixed and slides made as described above for testis cells.

Results and Discussion

The physiological temperature range of the echidna lies somewhere between 25°C and 30°C. Above 30°C its temperature rises with the environment. In the absence of efficient sweat glands, panting mechanism, and heat loss by vaso-dilation of surface capillaries, the animal dies of heat stroke at 37°C if unable to burrow[12,13]. The echidna caught in winter had a cloacal temperature of 29°C whereas the one found in summer had a temperature as high as 33°C.

The results of the temperature tests for the echidna leucocyte cultures are given in Table I. The concentration of colcemid used was much higher than that usually employed for leucocyte cultures of eutherian mammals and approaches the concentrations that have been used successfully for amphibian cells[14]. The best results were obtained with a final colcemid concentration of 0.01%. The concentration of PHA is also higher

than that used for mammalian leucocytes and approaches those that were successfully used to culture amphibian leucocytes.

Echidna cultures kept at 37°C showed no divisions after 72 hours; after 5 days at this temperature, the nuclei remained small, and a number of them became pycnotic. The best results were obtained from cultures kept at 27°C. Leucocyte cultures of eutherian mammals and those of marsupials grown at 37°C show a mitotic peak after 72 hours. At a temperature 10 degrees lower, one would expect, because of lowered metabolism, that the onset of the mitotic wave would be delayed; thus it is not surprising that for echidna leucocytes the highest mitotic index was obtained around the 7th day of culture. However, there appears to be no sharp peak in the number of mitoses. Forty-eight hours of colcemid treatment is necessary to obtain a reasonable mitotic index, and a high level of divisions is maintained for the following 48 hours.

The chromosome number in the two male echidnas examined was found to be 63 and in the single female 64. Chromosome counts were obtained from testis preparations and leucocyte cultures in the case of the male and from leucocyte cultures for the female echidna. At least 20 metaphases which gave these numbers were scored for each sex.

Metaphases obtained from leucocyte cultures gave many suitable spreads (Fig. I). Drawings from projected photographic negatives of echidna chromosomes are shown in figures II and III. With long colcemid treatment, the chromosomes become contracted and the chromatid arms are well separated at the time of fixation. The position of the centromeres

Table I

Mitotic Indices in Cultures of Echidna Leucocytes

Temperature	Colcemid Treatment (hours)	Culture Time (days)	Mitotic Index (Metaphases/1,000 cells)
27C	2	3	none
27C	2	6	none
27C	48	6	mainly prophases
27C	58	6	1 - 2
27C	48	7	5 - 20
27C	48	7	>20*
27C	48	8	5 - 20
27C	48	9	5 - 20
35C	48	5	1 - 2
37C	24	3	none
37C	48	5	none

* Final concentration of colcemid was 0.01% in this treatment compared with 0.007% in all other series.

could thus be determined with accuracy.

From figures IV and V it can be seen that the largest chromosomes of the echidna complement are acrocentric. The pair in position 5 possesses two satellites separated from the centromere by a long hetero-

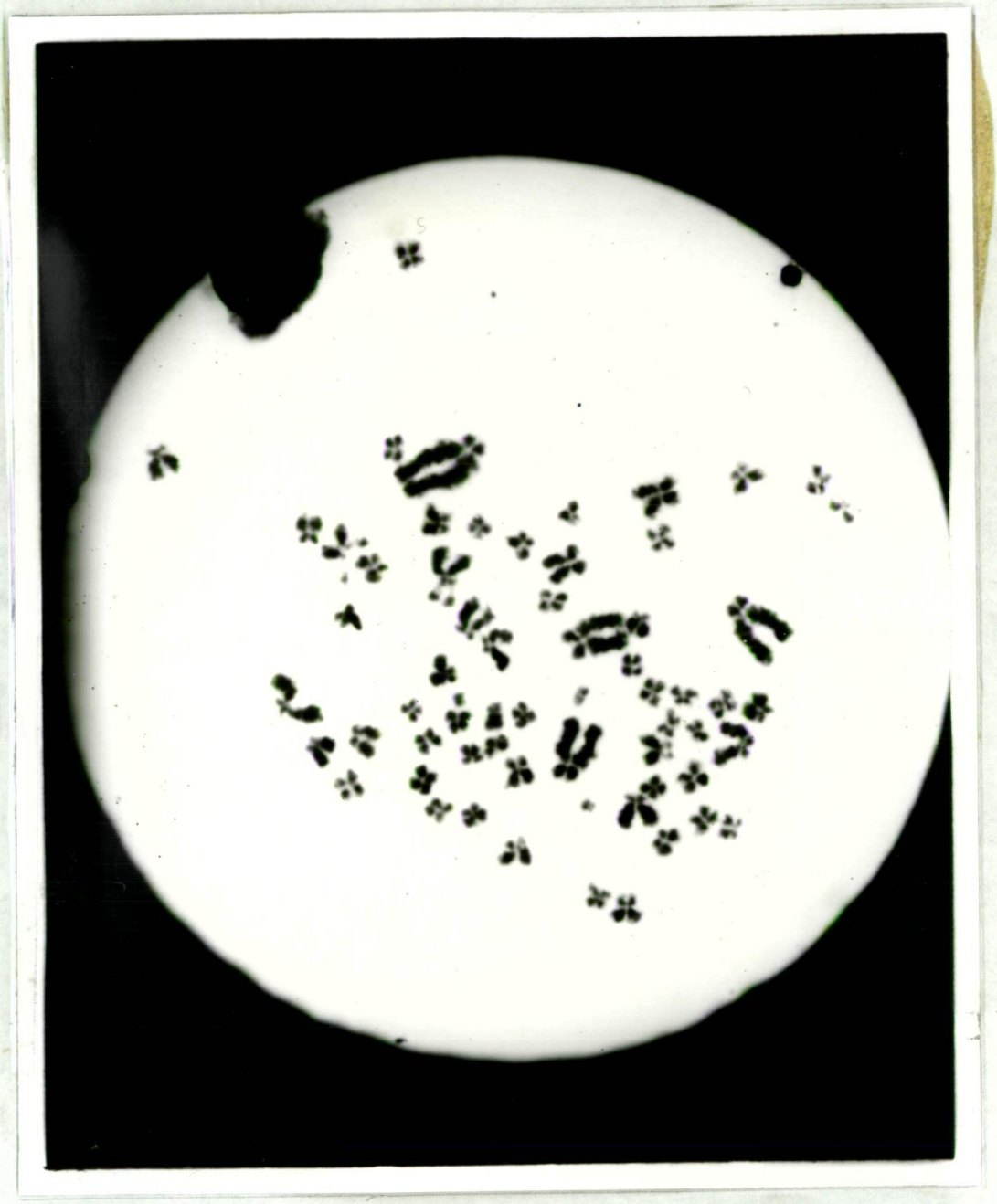

Fig. I. Metaphase chromosomes from female echidna obtained from leucocyte culture

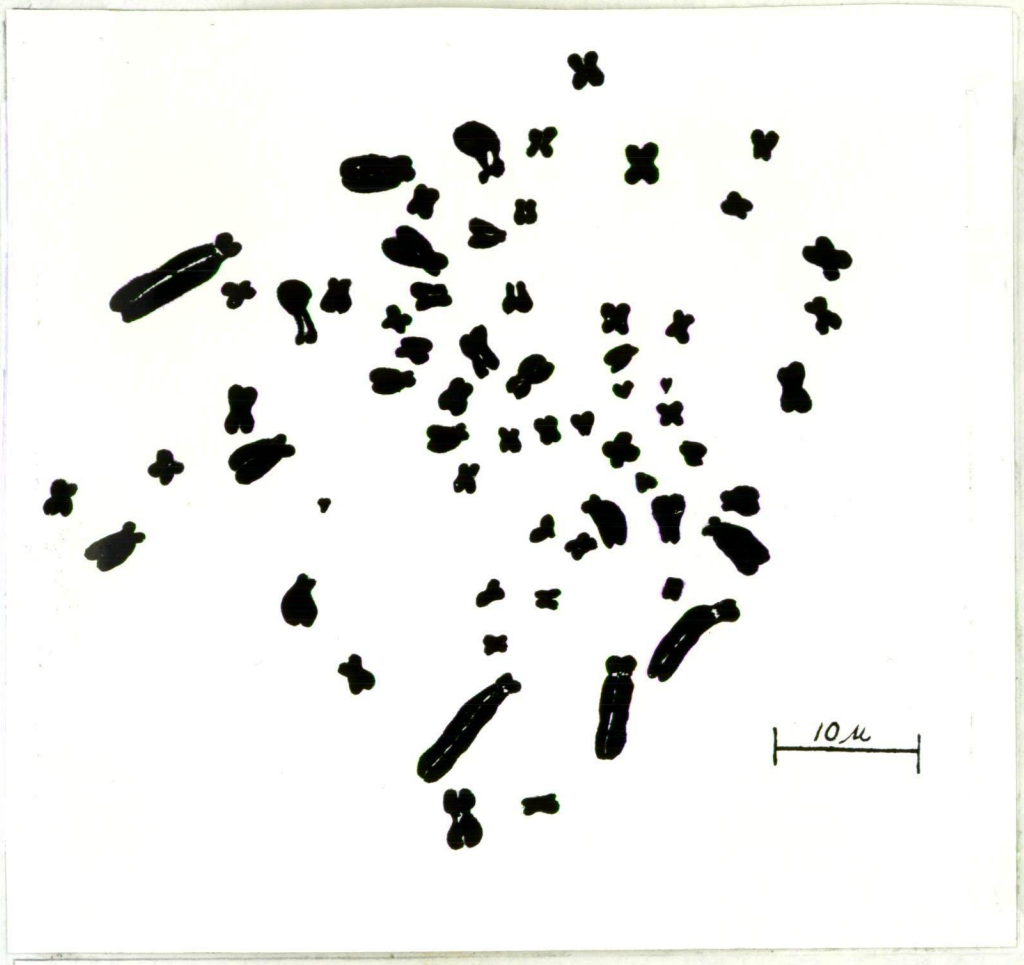

Fig.II. Metaphase chromosomes of a male echidna obtained from testis without any colchicine treatment.

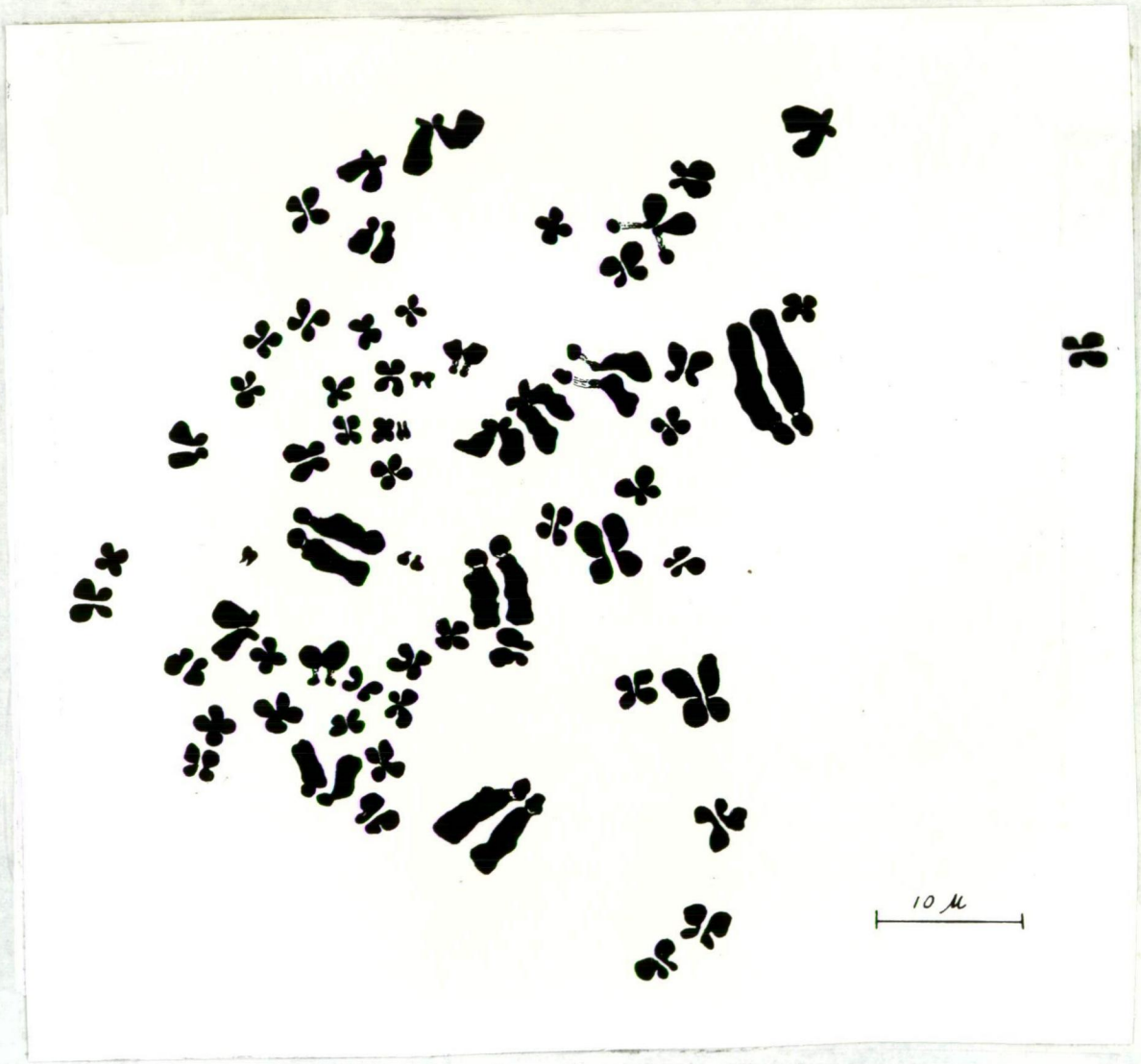

Fig.III. Chromosomes of female echidna from leucocyte culture with 48 hours colcemid treatment.

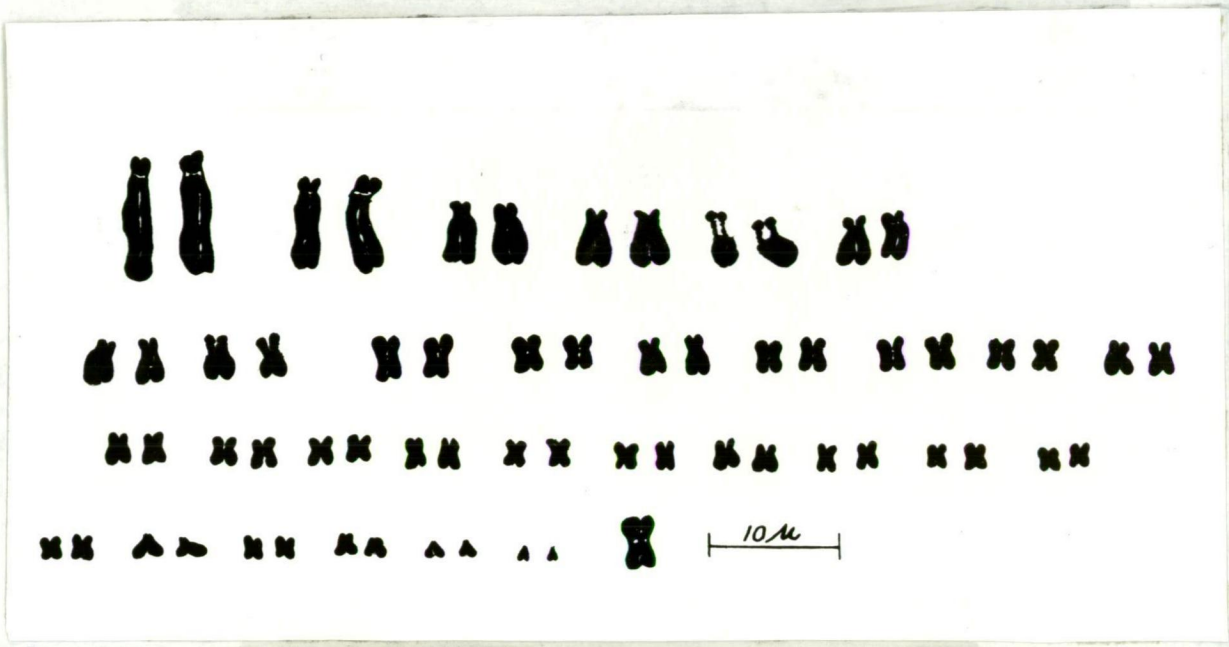

Fig.IV. Idiogram of male Echidna: 31 pairs of autosomes and one X chromosome

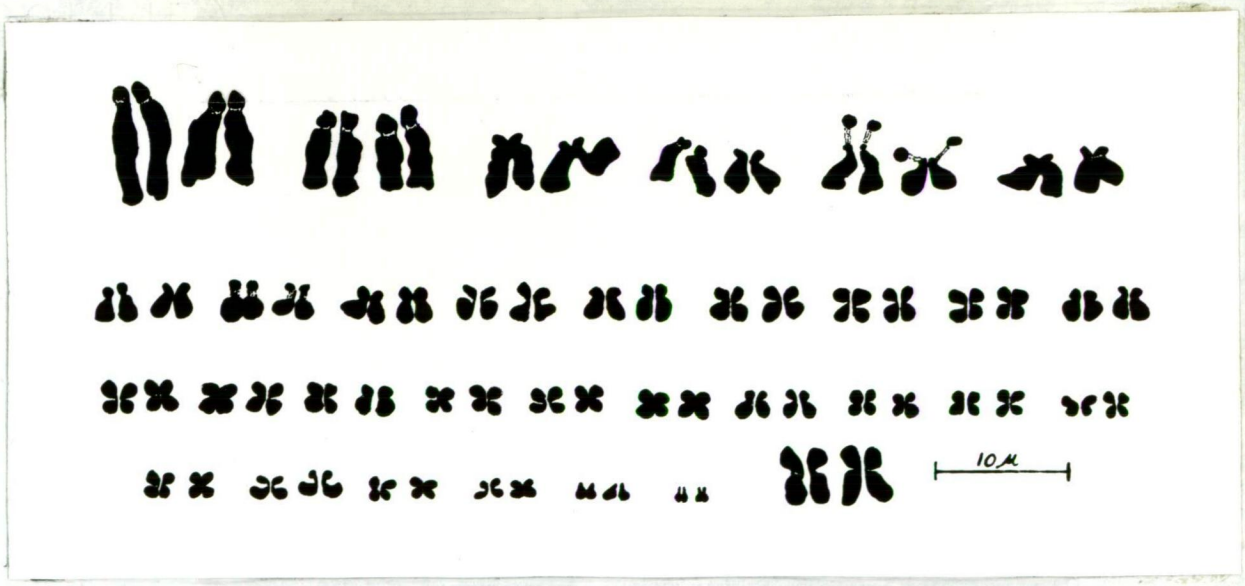

Fig.V. Idiogram of female Echidna: 31 pairs of autosomes and two X chromosomes

chromatic segment. This pair of chromosomes is easily recognised, although sometimes the heterochromatic region may be relatively condensed and shortened. The pair in number 8 position has a similar pair of satellites; but the heterochromatic region is shorter relative to the other arm of the chromosome, and the satellite bodies are not as distinct as in the former pair.

The chromosomes from pair number 9 to pair 26 are metacentric or sub-metacentric, gradually decreasing in size, often by so little that there is difficulty in pairing the homologues. The smallest pairs, 30 and 31, are much smaller than the rest of the chromosomal complement. The pair number 31 is about half the size of the pair 30. Both the smallest pairs appear to be telocentric or subtelocentric.

In the male echidna there is one large metacentric chromosome which has been called the X chromosome. This is easy to recognise as it is the largest metacentric chromosome present. In the female echidna, there are two of these chromosomes. (It is evident that the large acrocentric thought to be the sex chromosome by Van Brink[3] is the largest autosome.)

The platypus leucocytes divided at 27, 31, 35 and 37°C. Unlike echidna leucocytes, platypus cultures at 37°C had a good mitotic index after 3 days in culture and only 4 hours colcemid treatment. Cultures at 27°C showed divisions only on the 7th to 9th day (Table II) as in the case of echidna. Leucocyte cultures from the platypus gave good mitotic indices with 2 per cent PHA as well as higher concentrations. Mitosis

Table II

Mitotic Indices of Platypus Leucocyte Cultures

Temperature	PHA	Colcemid Treatment (hours)	Culture Time (days)	Mitotic Index Metaphases/1,000 cells
27C	5½%	20	6	none
27C	5½%	40	7	5 - 10
27C	2%	20	8	5 - 10
27C	5½%	20	8	5 - 10
27C	5½%	20	9	5 - 10
31C	5½%	4	4	1 - 5
31C	5½%	20	4	10 - 20
35C	5½%	20	3½	1 - 2
37C	5½%	4	3	10 - 20

Final concentration of colcemid was 0.01% in all cultures

also occurred when 0.4 ml of whole blood was added to the culture instead of washed leucocytes. This suggests the absence from the plasma of the platypus of a strong antimitotic agent which occurs in the plasma of some mammalian species[15].

The leucocyte cultures of the platypus gave chromosome counts of 53 for the male and 54 for the female. The spleen of the female also gave counts of 54. Drawings from projected photograph negatives of the

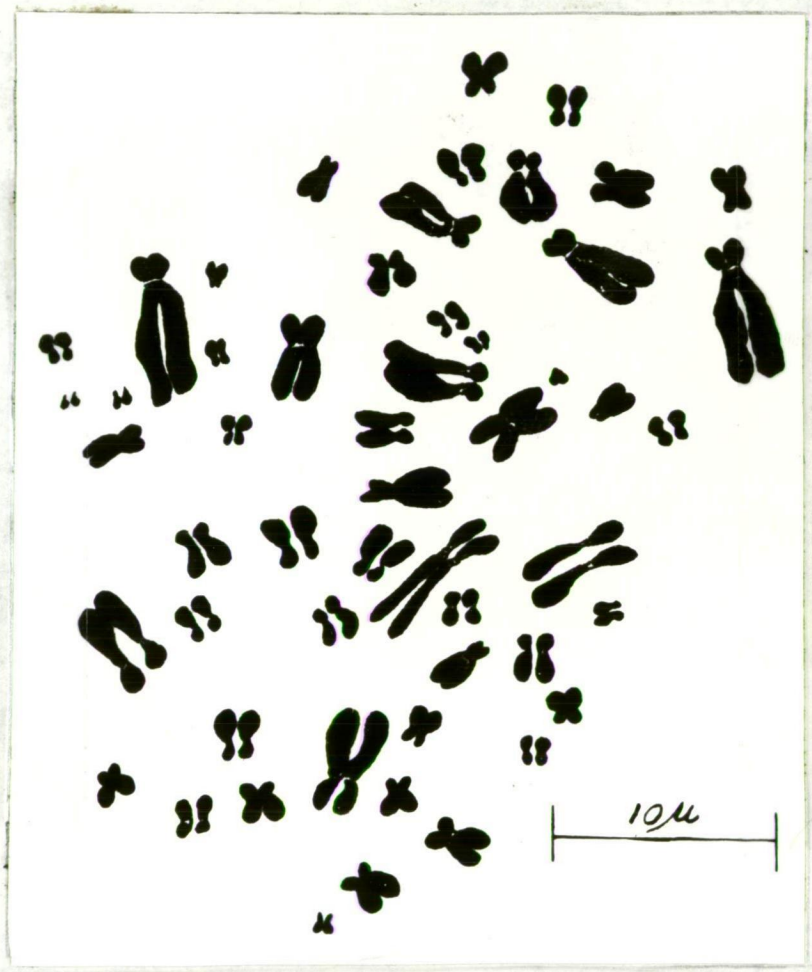

Fig.VI. Chromosomes of the male platypus from leucoctye culture, 20 hours colcemid treatment.

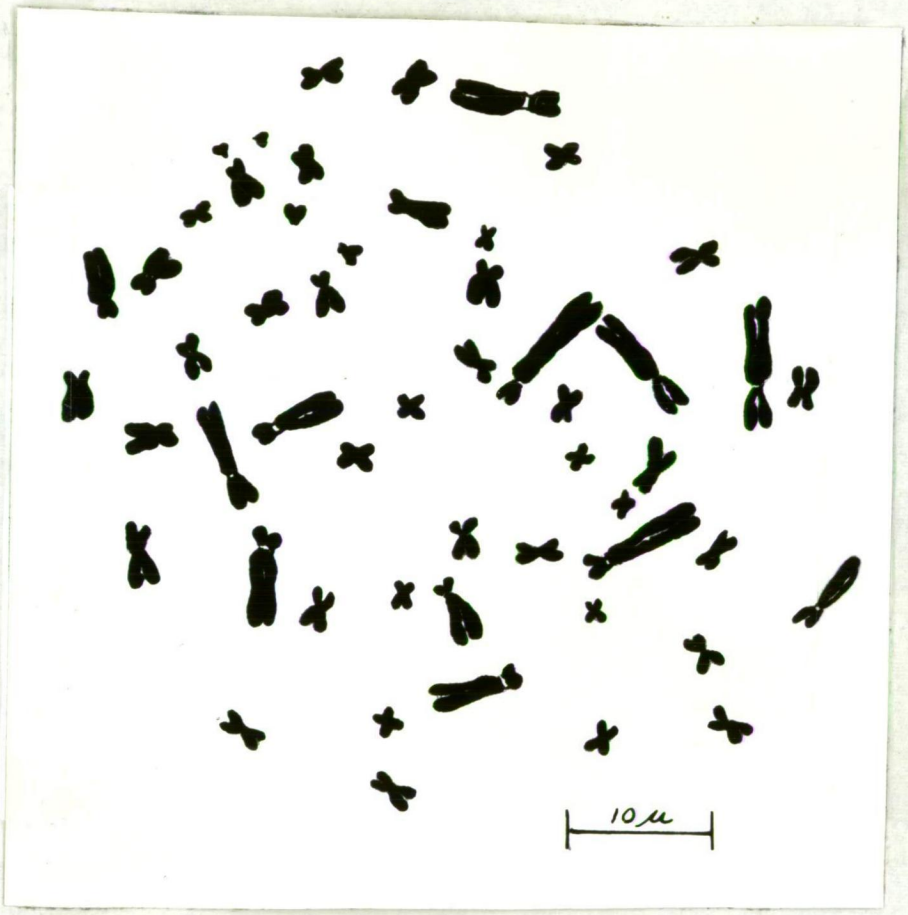

Fig.VII. Chromosomes of the female platypus from the spleen which was removed 2 hours after the animal was injected with colcemid

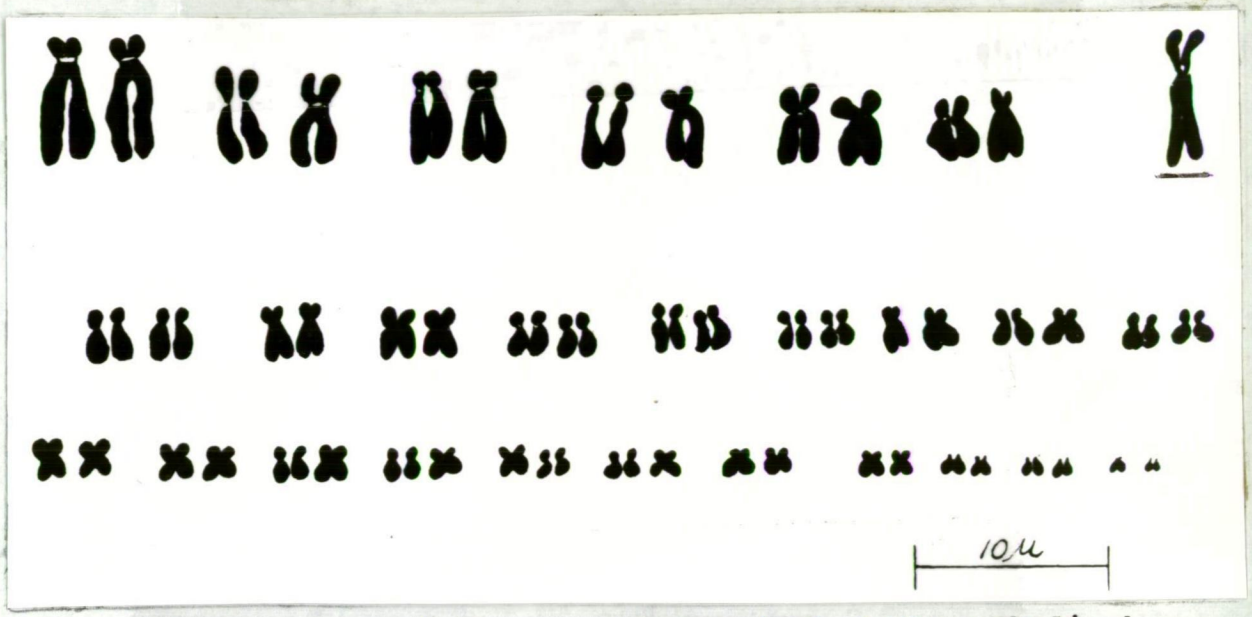

Fig.VIII. Chromosomes of the male platypus, X chromosome underlined.

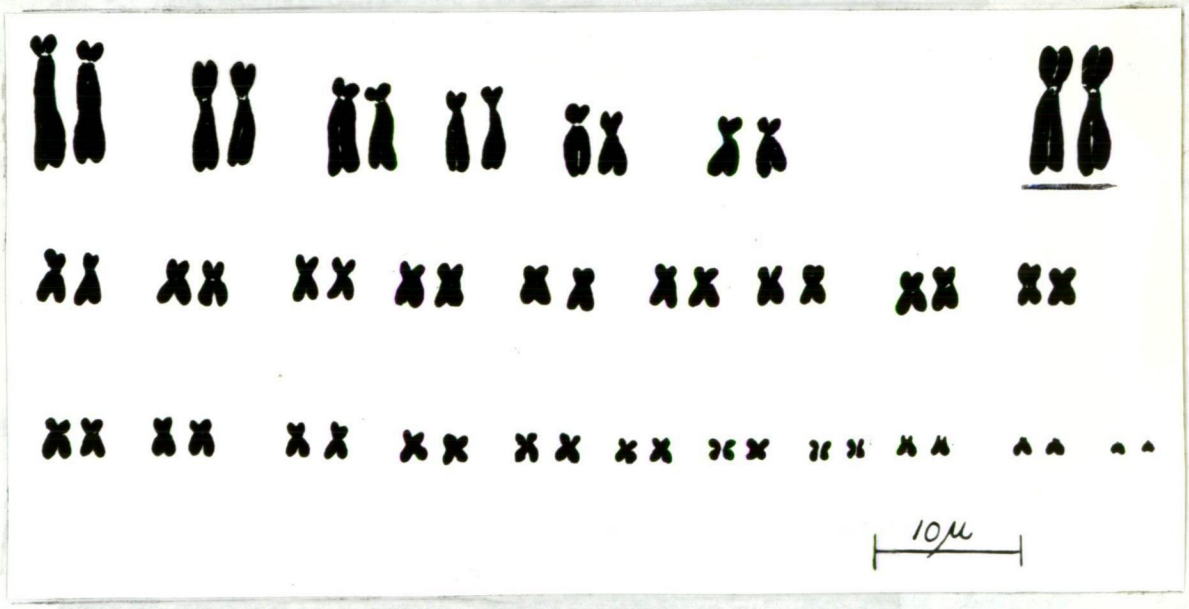

Fig. IX. Chromosomes of the female platypus, X chromosomes underlined.

chromosome spreads obtained from male and female platypus tissues are shown in figures VI and VII.

From figures VIII and IX it can be seen that the male platypus like the male echidna has six pairs of large chromosomes; the second largest pair is, however, submetacentric and not acrocentric as in the echidna. The smaller elements of the platypus complement are metacentric or submetacentric, gradually decreasing in size down to the three smallest pairs of chromosomes which are telocentric or subtelocentric. The smallest pair, number 26, is about half the size of the pair number 25.

The large unpaired submetacentric chromosome in the male platypus which we have designated as the X chromosome (there are two of these in the female platypus) is somewhat larger than the corresponding X chromosome of the echidna. This X chromosome of the platypus has a slightly heterochromatic region adjoining the centromere in the shorter arm, and this part of the chromosome often appears differentially condensed to the rest of the chromosome complement.

Conclusion

In the echidna, the chromosome numbers are 64 in the female and 63 in the male, and in the platypus 54 in the female and 53 in the male: thus unlike the situation in Aves and Ophidia the monotremes resemble all other known mammals in having male heterogamety.

It is possible that both monotremes represent some form of mosaicism similar to that in the creeping vole[16]. However, in the case of the echidna, examination of the testis seems to preclude this possibility

since the chromosome number obtained from testicular mitoses was 63, the same as in leucocyte cultures of the male. In the female platypus the chromosomal number was 54 both from spleen preparations and leucocyte cultures.

The chromosome numbers of both monotremes strongly suggest an XX-XO sex-determining system of which no example has so far been recorded among the mammals[17]. A careful examination of meiosis would be necessary to decide this point, but this is rendered difficult by the extremely short seasonal meiotic activity in these animals. Possibly the sex-determining system of the monotremes is more complex, similar to the $X_1X_1X_2X_2/X_1X_2Y$ system found in two placental mammals[8,9] and in one marsupial[10]. It is of interest that recent work has shown that male heterogamety exists in two different genera of Lacertilia and the sex-mechanism is of the $X_1X_1X_2X_2/X_1X_2Y$ type[18,19]. Autoradiographic investigations of blood cultures of the monotremes would help to decide the question.

Both White[20] and Matthey[2] have noted a resemblance between the chromosome morphology of the monotremes and that of birds and reptiles. The chromosomes of echidna and platypus do in fact show a marked range in size, but there is a gradation, and even the smallest chromosomes are considerably larger than the microelements typical of the birds. All chromosomes have a well defined centromeric constriction. It would appear from comparison with the work of Beçak et al.[21] that the monotreme karyotype may represent an extreme mammalian or Chelonian type rather than one with affinity to that in Aves and Ophidia.

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D. A comparison of the DNA content of the monotremes with that of placental mammals and marsupials

Much interest has recently been focused on the DNA content of the different classes of vertebrates and on the basic cytological relationship within the animal kingdom. Although the diploid number and size of chromosomes varies considerably in placental mammals, biochemical estimations[1] showed that the DNA content was relatively constant. Atkin et al.[2], using the microdensitometer method, found that the placental mammals constitute a fairly uniform group with regard to the total genetic content, and that the birds also show a uniformity with a DNA content about half (44 to 59%) the eutherians. The reptiles, however, fall into two distinct categories[2]: the order Squamata has a DNA value 60 to 67% that of placental mammals, while Crocodylia and Chelonia have 80 to 89%. These percentages agree with those obtained by Ohno[3,4] who measured chromosomal areas of colchicinized metaphases.

The difference between the DNA content of the placental mammals and birds suggests that these two classes of warm blooded vertebrates have evolved from different lineages; however, the extensive speciation within these two classes was carried out without substantial change of the genetic content. It would be of interest to know where the other two groups of mammals, the marsupials and monotremes, belong. No information is available for the monotremes, but Ohno[4] obtained for the American opossum (Didelphis virginiana) by chromosomal area estimations a value

of 94% that of placental mammals.

In this section, the DNA content and relative chromosomal area was investigated for different groups of mammals. The estimation of DNA values for heart tissue by the biochemical method of Schmidt and Thannhauser[5,6] is described for two monotremes, the platypus (Ornithorhynchus anatinus) and the echidna or spiny ant-eater (Tachyglossus aculeatus); the marsupial potoroo (Potorous tridactylus), and the rat (Rattus norvegicus) as a representative of placental mammals.

Following Ohno's technique[3], a preliminary estimation was made of relative chromosomal areas from colchicinized cells in leucocyte cultures of man, potoroo and echidna. The values obtained for the area of the echidna chromosome complement relative to that of man was in good agreement with the relative values of DNA content of echidna and rat obtained by biochemical estimations.

Ohno et al.[3] have shown that in spite of the variety of diploid chromosome complements encountered in placental mammals, not only is the total mass of chromosomal material constant, but the X chromosome of different species is almost identical in absolute size. The great majority of placental mammals, including man and mouse, contain what these authors came to call the original-type X chromosome. In the golden hamster the X chromosome is about twice that size, and in the creeping vole, about three times; these were termed respectively by these authors the duplicate-type X and the triplicate-type X. Using the same techniques as employed by Ohno, the size of the chromosome

designated as the X-chromosome in the echidna (described in section C of this thesis) was calculated.

Materials and Methods

DNA content by the method of Schmidt and Thannhauser[5,6,7]

The material used was heart ventricles and in the case of the rat two heart ventricles were used for each determination, so that the weights employed were approximately the same as for the other animals. Heart was adjudged the most suitable for the purpose since it is one of the organs least likely to be affected by variations in the condition of the animal, and the ratio of the nucleus to the cytoplasm remains fairly constant even for animals in poor condition; moreover,heart tissue does not present the problem of polyploidy as in the case of the liver.

(a) Homogenization

The heart tissue was weighed quickly and kept for 24 hours at -15°C. This greatly facilitated the subsequent homogenization. The material (in cold room, 0° to 2°C) was first finely sliced with a razor and then homogenized in a Braun homogenizer with glass-distilled water (approximately 1.5 grams heart tissue with 15 ml water).

(b) Removal of acid soluble compounds (cold room temperature of 0° to 5°C)

1 ml of tissue homogenate was mixed with 2.5 ml 10% trichloracetic acid (T.C.A.) and centrifuged (600 g for 20 minutes). The residue was washed in 2.5 ml T.C.A., centrifuged and the supernatant removed.

(c) Removal of phospholipides (room temperature of 25°C)

The residue was resuspended in 1 ml distilled water and 4 ml of 95% ethanol were added. [Very careful mixing with a fine glass rod was carried out throughout the whole procedure. The time taken for each resuspension was 5 to 6 minutes with careful breaking up of pellets until an even powdery suspension was obtained.] After centrifugation the residue was washed three times with careful stirring with ethanol-ether mixture (3:1).

(d) Removal of RNA

Alkaline digestion at 37°C:-

The residue was dispersed in 2.0 ml of 0.3N aqueous potassium hydroxide, the tube covered and incubated for 20 hours at 37°C.

Neutralization and acid digestion at 0° to 5°C:-

The KOH was carefully neutralized with 6N HCl using an external indicator and dropping the HCl with a fine Pasteur pipette. Stirring and testing the pH was carried out between the addition of each drop. After centrifugation, the residue was washed with 5 ml of 5% perchloracetic acid (P.C.A.).

(e) Removal of DNA

Perchloric acid digestion at 90°C:-

The residue was heated with 5 ml of 5% PCA for 15 minutes in a water bath at 90°C, then the mixture was cooled to room temperature and centrifuged. The supernatant (containing DNA-P fraction) was carefully transferred to a fresh tube and the residue washed again (with warming on the water bath) with 2.5 ml PCA. The supernatant was added to the first

washing, and the combined DNA fraction was made up to 10 ml and mixed well.

(f) UV estimation of DNA-P

One ml portion of the combined DNA fraction was made up to 50 ml with 5% PCA, and absorbances were read for samples and blanks at 260 millimicrons with a Unicam spectrophotometer (model SP500). The concentration of DNA in the sample is found from the following equation:

$$\varepsilon(P) = \frac{30.98E}{c \times \ell}$$

The symbol $\varepsilon(P)$ is used for the atomic extinction coefficient with respect to P as defined by Chargaff and Zamenoff[7] and $\varepsilon(P)$ at λ max = 7300.

E is the extinction coefficient ($\log I_o/I$)

c is the concentration of P in g/litre

ℓ is the thickness of absorbing layer

Since $\ell = 1$,

we have $c = \frac{30.98 \times \text{dilution} \times \text{UV reading at 260 m}\mu}{7300}$

= concentration of DNA in undiluted sample in grams/litre

Chromosomal area estimations

Mitotic figures were obtained from human, potoroo and echidna leucocyte cultures. Colcemid treatment was applied for the final 2 hours of culture in case of the potoroo, 17 hours in case of human leucocytes and 48 hours in echidna cultures. All cells had been given

10 minutes hypotonic treatment, fixed in glacial acetic ethanol mixture and stained with Leishman's stain. Only those cells were selected in which every chromosome was (1) evenly stained (2) sharply outlined (3) clearly separable into two sister-chromatids and (4) lying flat on the optical plane. Nuclei were chosen in which the chromosomes showed a medium amount of condensation - the highly condensed and contracted chromosomes which appear after a few hours of colchicine treatment were avoided.

Photograph plates of 5 metaphase figures were selected for each species. Each negative was placed in a photographic enlarger and the image projected onto a sheet of white paper (Heralds Loan) at a final magnification of x3,000. The outline of each chromosome was carefully traced with a sharp hard pencil, and the images cut out and weighed on a precision balance.

Several paper cutouts of 50μ x 50μ thrown onto the paper at the same magnification were weighed and the average of these taken as the standard weight for $2{,}500\mu^2$. By the use of this standard the chromosomal areas of the diploid complement were obtained from the weights of the cutout chromosomes.

Ohno[3] had noticed that the two X chromosomes within the same female metaphase differed often by as much as 20% in weight; consequently the value for X was obtained for each metaphase by taking the mean of the two Xs. The percentage of X in the haploid set, i.e. $\frac{X}{A + X}\%$ was calculated individually for each of five cells and the arithmetic mean was determined.

The weight of the haploid set of autosomes was calculated as follows:-

for ♀ , $A = \frac{\text{total weight of chromosome complement - weight of 2Xs}}{2}$

Results and Discussion

The results of the DNA content found for the rat, potoroo, echidna and platypus are given in Table I.

Table I

DNA content expressed in mg/100 g of fresh heart tissue

		$\bar{x} \pm S\bar{x}$	Relative content
♂	Rat	29.9 ± 0.24	100%
♂	Potoroo	24.2 ± 0.20	81%
♂	Echidna	27.9 ± 0.18	93.3%
♀	Platypus	29.5 ± 0.27	98.6%

Each DNA content given is the mean of four separate estimations

The higher DNA value for the female platypus compared with the male echidna is probably due to the presence of the extra sex chromosome.

The results show that the DNA content of the potoroo is about 10% lower than that of the American possum, the only other marsupial which appears to have been examined[4]. The difference may be associated with the fact that the potoroo has only about half the number of chromosomes (12 in ♀ ; 13 in ♂) of the opossum (2n = 22) and one may compare it

with the 10 percent difference in DNA value between the creeping vole (Microtus oregoni 2n = 17-18) and other placental mammals[2]. Atkin et al.[2] have suggested that the low number of chromosomes in the vole is associated with the loss of a number of centromeres with their adjacent heterochromatic chromosomal material. In the case of the potoroo, Sharman and Barber[8] have shown that autosomal material has been incorporated in the sex system of the animal.

The technique of Ohno[3] was used in a preliminary survey of the genetic content of three groups of mammals (man, potoroo, echidna, table II). By comparing chromosomal areas of leucocytes in tissue culture, it was found that the chromosomal area of the echidna was 92.9% that of the rat. This is in good agreement with the biochemical estimations of the DNA content which gave a value of 93% for the echidna compared with that of the rat.

Table II

Average chromosomal area of the diploid complement found from colchicinized leucocyte cultures

	Average Area	No. of Metaphases scored	Relative Content
♂ human leucocytes	341μ^2	5	100%
♀ potoroo leucocytes	285μ^2	5	84%
♀ echidna leucocytes	318μ^2	6	92.9%

The results of the above table have been calculated from photo-

graphic negatives of five to six metaphases, the only ones available at the time. The accuracy of the chromosomal area estimations could be increased by scoring larger numbers of metaphases.

Ohno[3] has pointed out that the estimation of the amount of genetic material contained in a diploid complement by weighing paper cutouts of colchicinized metaphase chromosomes is admittedly wanting in refinement, but on the other hand neither biochemical nor cytochemical methods for estimating DNA content produce more precise results[3]. Recently the microdensitometer method employed by Atkin et al.[2] has been criticised on the grounds that the Feulgen stain can vary from nucleus to nucleus by quite large amounts for the same species[9]. With the present techniques available, it is difficult to be certain of the absolute value of tissue and cellular content of DNA. However, in case of the biochemical estimations reported in this section, all experiments were carried out in a similar manner and with the same chemicals and standards. Thus these results should reflect fairly accurately the relative DNA content of the four species in question. The DNA value of 29.9 mg/100 g of fresh heart tissue obtained for the rat is in good agreement with the value of 30.6 mg/100 g of rat heart quoted by Chargaff and Davidson[10].

In estimations of the size of the X chromosome, Ohno et al.[4] found that in placental mammals the value for $\frac{X}{A + X}\%$ ranges from 5 to about 6.45%. For birds these authors found the $\frac{Z}{A + Z}\%$ to be of the order of 7 to 9 per cent.

The few estimations carried out for the echidna gave for $\frac{X}{A + X}\%$

the following values: 5%, 6%, 5% and 5.9% (average 5.5%). Thus in this respect it would appear that the echidna lies closer to the placental mammals than it does to the birds.

Conclusion

The monotremes have been compared with the reptiles because of certain anatomical and physiological similarities in these two groups of vertebrates[11,12]. The chromosome complements of the monotremes have been described as resembling those of snakes and birds because of the presence of macro and micro elements. However, from the experiments carried out in sections C and D of this thesis, it would appear that in some respects the monotremes are more closely allied to the placental mammals than to birds or snakes[13,14,15]. Unlike birds, but similar to mammals, the male of the monotremes is the heterogametic sex. If the chromosome designated here as the X chromosome of the echidna is the original X and has not been altered by any inclusion of autosomal material by translocation, then the $\frac{X}{A + X}\%$ value falls well within the range obtained for the placental mammals and is quite distinct from that of the birds. The biochemical estimations of the DNA content for the platypus and echidna have again shown them to be closer to placental mammals and marsupials than to other vertebrates. Even the monotreme karyotype can be regarded as an extreme mammalian or Chelonian type rather than resembling those of Aves and Ophidia.

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E. Effects of Heliotrine on the Mitotic Index and Chromosome Breakage in Marsupial Cells *in vitro*

Introduction

Chemical substances have been known for some time to arrest mitosis and induce tumours in the same way as ionising radiations[1]. Since the discovery that mustard gas and its analogues caused mutations (Auerbach and Robson[2], 1943) followed by Koller's[3] observation that the same chemicals induced chromosome aberrations similar to those produced by Xrays, many substances have been found to possess radiomimetic properties.

Radiomimetic substances, like Xrays, have a selective action on tissues containing rapidly dividing cells. The first tissues to be affected are the blood-forming centres such as bone marrow, the genital organs, the expendable linings found in the stomach and intestines, all places where cells divide rapidly and the mitotic index is high[4]. The pigment-forming cells of the hair are sensitive to both X-irradiation and radiomimetic substances and local greying of the hair occurs at the site of the injection of these chemicals.

Besides acting preferentially on actively proliferating cells of the animal, radiomimetic substances, like Xrays, destroy lymphocytes at very low doses (as low as 10^{-5}M or less *in vitro*[5]). They can also be utilized instead of Xray therapy to cure malignant tumours, but unfortunately the similarity extends to their ability to produce cancer.

Mustard gas and nitrogen mustard have been known to produce malignant tumours in experimental animals after long periods of administration[4].

A wide variety of substances will inhibit cell division at very low concentrations. Dustin[6] has divided them into two groups: (1) agents that act during mitosis by destroying the spindle and arresting the cell at metaphase (e.g. colchicine which acts at a concentration of 10^{-5}M on both plant and animal cells *in vivo* and *in vitro*), and (2) agents that act on the nucleus during the resting stage and prevent the onset of mitosis. Radiomimetic substances fall into the latter category. This radiation-like effect on cell division differentiates these substances from ordinary chemical poisons which kill the cell at the time of administration[4].

Like mustard gas and its analogues, many of the radiomimetic substances are alkylating agents which can transfer alkyl groups ($-CH_3$, C_2H_5, C_3H_7 etc.) to other compounds. Recently the pyrrolizidine alkaloids, many of which can act as biological alkylating agents, have been added to the list of chemical mutagens with radiomimetic properties. These alkaloids have been known for many years to be potentially dangerous hepatotoxins to livestock. Two major alkaloids, heliotrine and lasiocarpine, both present in the plant *Heliotropium europaeum*, have been shown to possess considerable biological activity[7-13]. They are mutagenic in *Drosophila*[14-17] and are capable of causing chromosomal breakage in *Allium* and *Vicia*[18,19]. Schoental and Magee[13] found that lasiocarpine is a potent liver carcinogen in the laboratory rat, and

Christie[20] and Gallagher[21] investigated some of the biochemical disturbances produced in liver cells *in vivo* and *in vitro*. There is still little detailed information, however, on the ability of these alkaloids to cause cytological damage in mammalian cells.

The following experiments were undertaken to investigate the radiomimetic properties of heliotrine in short-term cultures of peripheral leucocytes from *Potorous tridactylus*, the Tasmanian rat kangaroo. This marsupial has a small number of chromosomes: 10 autosomes, with 2 X chromosomes in the female and XY_1Y_2 sex chromosomes in the male[22]. The individual elements of the karyotype are readily distinguishable from one another, so that metaphase scoring is relatively easy since there is no doubt as to the identity of every chromosome. The favourable nature of this material for cytological work has already been pointed out by Sharman[23], Walen and Brown[24] and Shaw and Krooth[25].

Materials and Methods

The blood was obtained from the tail of the animal or by heart puncture using a syringe moistened with heparin. The blood was collected in a stoppered flask containing some heparin (2 drops for every 10 ml of blood), and air was blown through for 1/2 and hour while the blood was stirred mechanically with a magnetic stirrer. A couple of drops of Wellcome Phytohaemagglutinin (PHA) were then added to 10 ml aliquots of blood which were then centrifuged at 100 g for 5 minutes. The supernatant plasma containing the suspended leucocytes was centrifuged at 150 g for

7 minutes, the supernatant liquid removed and the cells washed twice with complete medium to remove any autologous serum and cultured by a modified method based on that of Moorhead et al.[26] using 12% foetal calf serum and 2% PHA. Higher concentrations, especially of horse serum, sometimes proved toxic to these marsupial cells although human leucocytes grew satisfactorily under similar conditions. Tissue culture medium 199 (Parker) and Hank's Balanced Salt Solution (B.S.S.) supplied by the Australian Commonwealth Serum Laboratories were used throughout.

Heliotrine was dissolved in sterile B.S.S. before being added to the cultures. In the controls, an equivalent amount of B.S.S. lacking the alkaloid was used. After incubating the cultures for 72 hours at 37°C, colchicine was added to give a final concentration of 0.004%. Two hours later, the cells were treated for 10 minutes at 37°C with hypotonic saline (1 of B.S.S.:2 of H_2O), then fixed with glacial acetic acid and absolute ethanol (1:3). Air dried films were rinsed in methanol and then placed in Leishman's stain for 10 minutes. To make sure that the metaphases scored were all first divisions, some of the cultures received colcemid (to give a final concentration of 0.0008%, i.e. 8µg/ml) at the 24th hour of culture. This is the time at which the first divisions appear in the potoroo leucocyte cultures (Figure 2). The cells were then harvested 60 hours after the initiation of cultures.

To compare the damage produced by heliotrine and by X-irradiation, experiments were carried out in which the fully oxygenated blood samples were exposed to Xrays delivered at a dose rate of 25r/minute (H.V.L.

0.5 m.m. Cu; 235 KV Peak). After irradiation, the leucocytes were separated and cultured as above.

Results

Heliotrine shows three easily observable effects on leucocytes in tissue culture:

(a) a variable despiralization of chromosome structure

(b) a suppression of mitosis

(c) chromosome breakage

(a) Despiralization of Chromosomes

In cultures containing heliotrine a number of cells in division showed secondary constrictions and a pronounced despiralization of chromosome material (Photo 1). Such metaphases were completely absent from the controls of these experiments which had received exactly the same treatment (except that instead of heliotrine solution an equal quantity of B.S.S. was added).

(b) Suppression of Mitosis

Cultures were terminated after 72 hours; the percentage of metaphases in controls was 4.5%. The effects of five different concentrations of heliotrine are presented in Table I. The dose-effect relationship for mitotic inhibition (figure I) appears to be sigmoidal up to a concentration of 1×10^{-5}M. Below 1×10^{-6}M, the effects of the alkaloid are very slight and in five replicates at 2×10^{-7}M no significant mitotic inhibition could be detected. This established a lower limit to the

Photo 1: Despiralization of chromatin matter and secondary constrictions in two of the chromosomes produced by heliotrine.

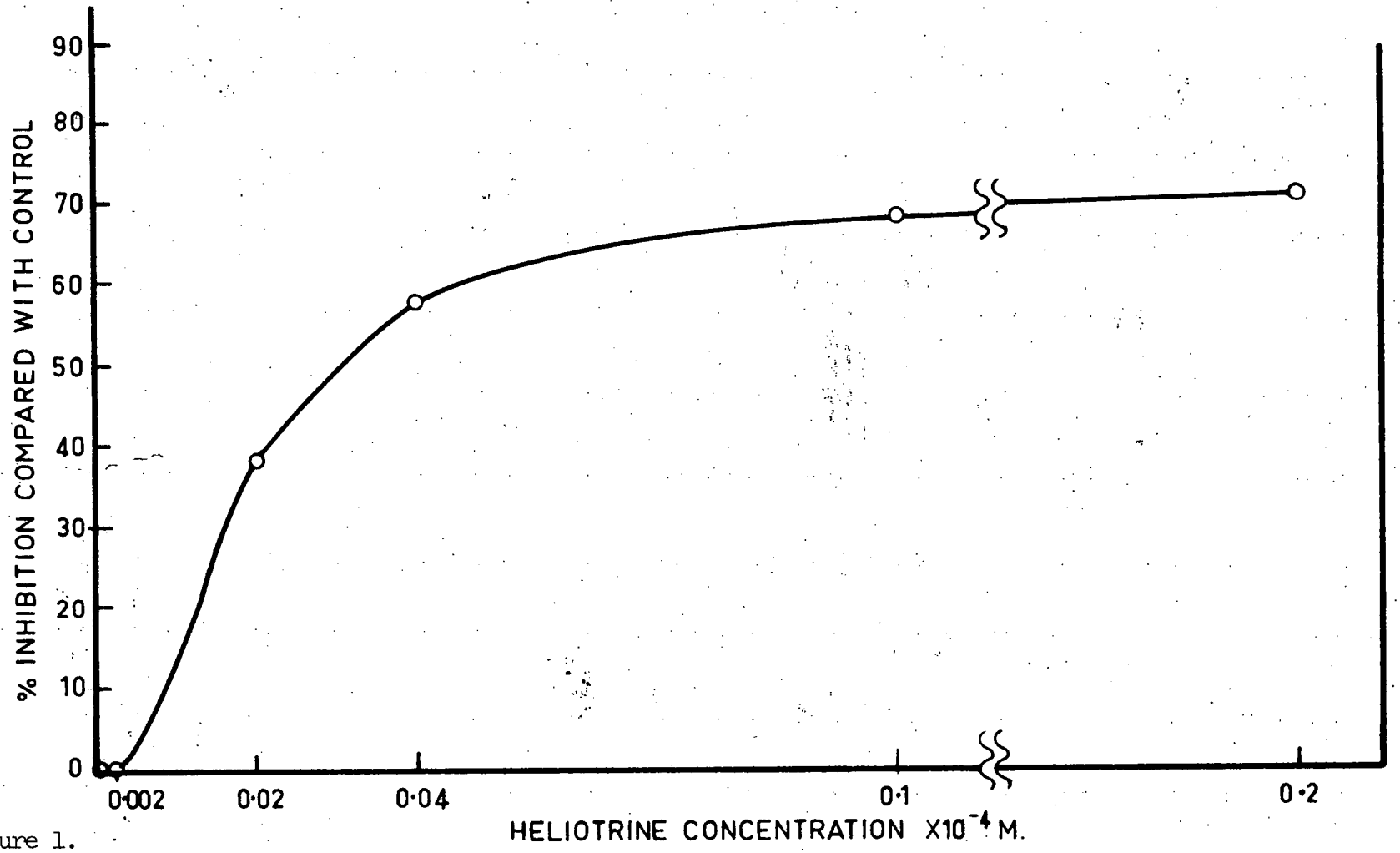


Figure 1.
Mitotic inhibition of potoroo leucocytes by heliotrine: colchicine treatment from 72nd to 74th hour of culture.

Table I

Mitotic Inhibition by Different Levels of Heliotrine

Heliotrine Conc.	M.I.%	M.I. as % of Control	% Inhibition	Cells scored
Control	4.5	100	0	7,000
0.02×10^{-4}M	2.8	62	38	7,000
0.04×10^{-4}M	1.88	42	58	7,000
0.1×10^{-4}M	1.46	32	68	7,000
0.2×10^{-4}M	1.3	29	71	7,000
2×10^{-4}M	<0.1	<1%	99%	7,000

All cultures received colchicine from 72nd to 74th hour

effective threshold concentration. To achieve nearly complete mitotic inhibition, it was necessary to increase the heliotrine concentration to 2×10^{-4}M at which level only a few prophases were seen when the cultures were harvested.

To determine the time of appearance of first mitoses and to estimate the rate of increase of the mitotic index in normal leucocyte cultures, two parallel cultures were set up. Samples were removed periodically and treated with colcemid for two hours. The cells received hypotonic treatment, were fixed and slides made as described above.

A few divisions appeared as early as 24 to 26 hours of the culture period (Table II), and then the mitotic index rose fairly sharply to give two peaks, one about the 60th hour and a second one around the 72nd (A & B, figure 2). A similar mitotic index curve showing two peaks was

Table II

Mitotic Indices in two Replicates of control Series at different culture Times with Colcemid Treatment for the final 2 hrs of each Period

Time of termination (in hrs) after start of culture	M.I.% - Control I	M.I.% - Control II
12 - 14 hrs	0	0
24 - 26 "	<0.01	<0.01*
36 - 38 "	0.2	0.3
48 - 50 "	1.2	1.0
60 - 62 "	1.9	2.2
66 - 68 "	1.7	2.0
72 - 74 "	2.3	2.4

*At 24 - 26 hrs there were only 1 to 2 metaphases in 10,000 cells; all other mitotic indices were estimated by counting 5,000 cells.

obtained by Michalowski[27] for cultures of human leucocytes.

In cultures of leucocytes with heliotrine added to give a final concentration of 5×10^{-5}M the mitotic index was considerably depressed (Table III). It can be seen from figure 2 that 5×10^{-5}M heliotrine suppresses the mitotic index appreciably: cultures stopped at the 48th hour of the culture period had a comparable M.I. with that of controls in the 38th hour, and for those stopped in the 71st hour, the M.I. is only that of controls in the 50th hour. Moreover, the M.I. of the controls has reached a peak by the 72nd hour, but in heliotrine-treated cultures it is still rising steeply. Thus 5×10^{-5}M heliotrine is

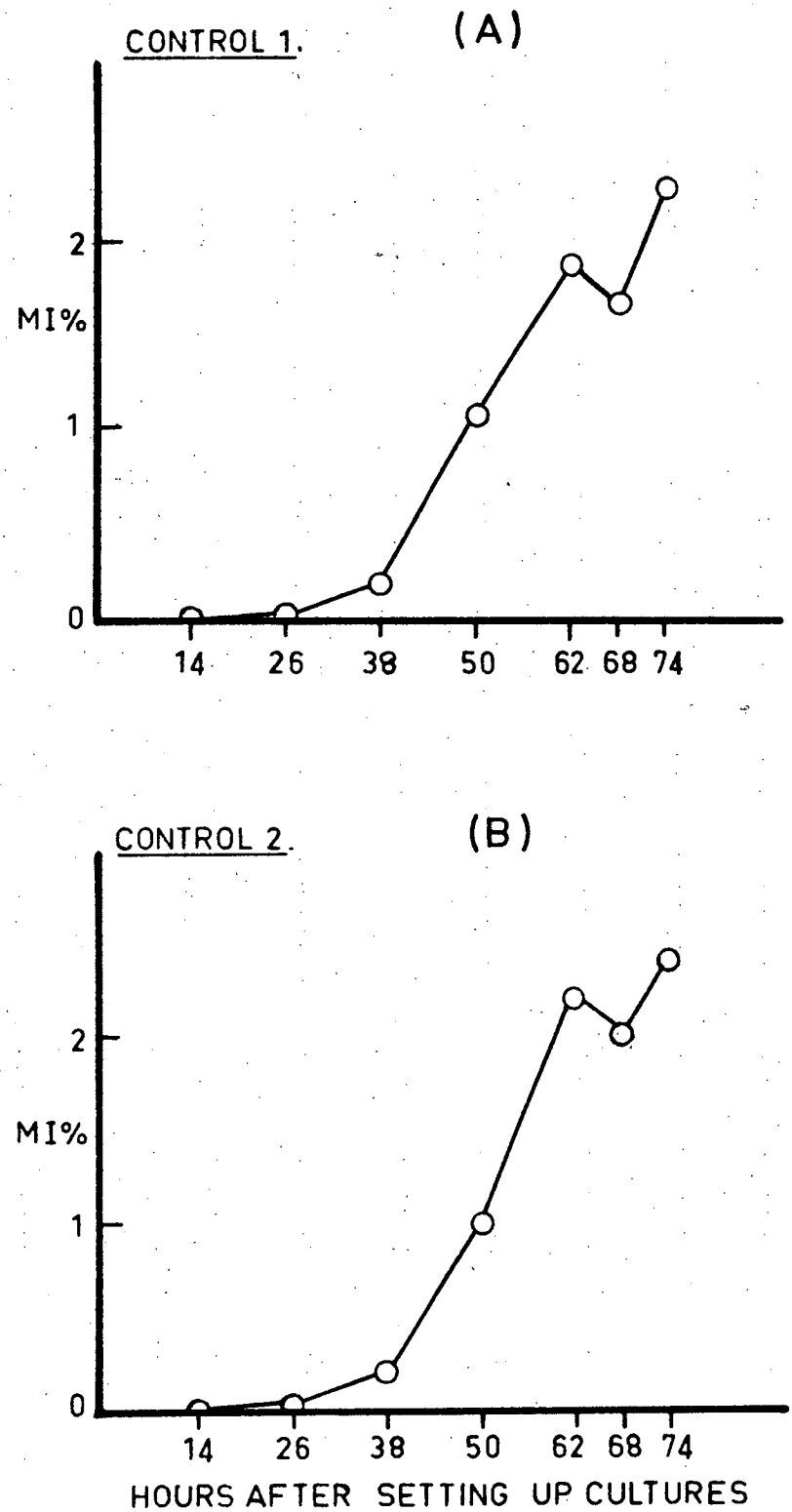


Figure 2. Mitotic index (in leucocyte cultures) relative to the period of incubation with and without heliotrine.

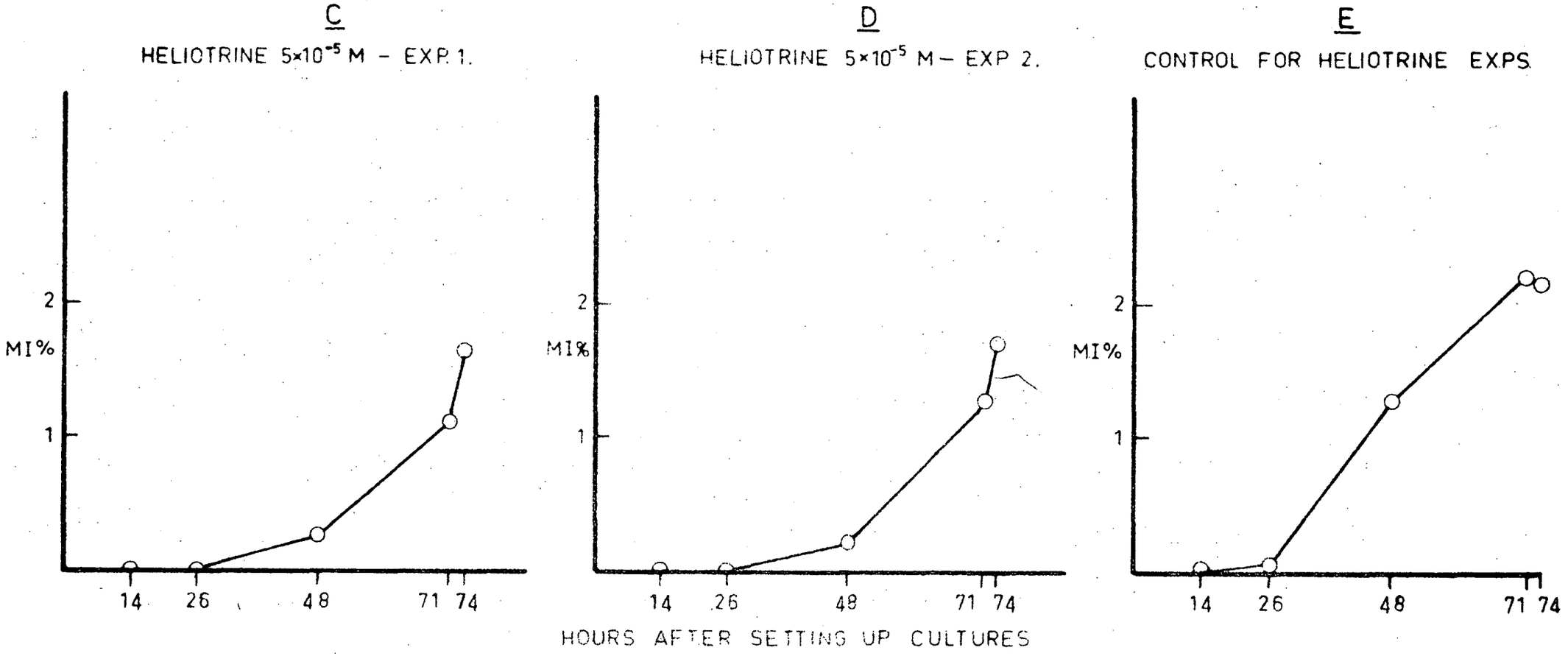


Figure 2. Mitotic index (in leucocyte cultures) relative to the period of incubation with and without heliotrine.

Table III

The effect of 5×10^{-5}M Heliotrine on the M.I. of Leucocyte Cultures; Colcemid treatment given for the final two hours of each culture period.

Time of termination (in hours) after the start of culture	M.I.%*		
	Heliotrine - exp. 1. 5×10^{-5}M	Heliotrine - exp. 2. 5×10^{-5}M	Control B.S.S. added instead of heliotrine
12-14 hrs of culture	0	0	0
24-26 " " "	0	0	1 → 2 metaphases/10,000 cells
46-48 " " "	0.26	0.20	1.26
69-71 " " "	1.10	1.28	2.20
72-74 " " "	1.65	1.70	2.16

* Each mitotic index is calculated for at least 5,000 cells

sufficient to cause a marked repression in the multiplication rate of leucocytes. This is reflected by a shift of the mitotic index with length of culture period so that at least a further period of 10 hours is required to achieve a mitotic level comparable with controls.

Cultures were treated with three levels of heliotrine concentration and colcemid added after 24 hours; the metaphases were scored at the 60th hour. Colcemid (like colchicine) arrests the cells at metaphase by destroying the mitotic spindle, and the addition of colcemid at the 24th hour of culture ensures that the cells are arrested in their first

division. The effects of different heliotrine concentrations on mitotic inhibition of first divisions occurring between the 24th and 60th hour of culture are given in table IV.

Table IV

Mitotic Inhibition of Leucocytes arrested in their first Division *in vitro* by three Levels of Heliotrine

Heliotrine Concentration	M.I.%	M.I. as % of control	% Inhibition	Cells scored
1.0×10^{-4}M	0.78	15.6	84.8	5,000
0.5×10^{-4}M	2	40	60	5,000
0.1×10^{-4}M	4.2	84	16	5,000
Control	5	100	0	5,000

Colcemid 24 hrs to 60 hours of culture period

(c) Chromosome Breakage

When heliotrine-treated cultures were terminated after 72 hours with colchicine treatment for a final two hours of the culture period, then the damage scored consisted of chromatid and iso-chromatid deletions as well as chromosome and chromatid interchanges (Photo 2), rings (Photo 3) and dicentrics (Table V). At concentrations above 1×10^{-4}M scoring became difficult because of the scarcity of metaphases, and after several attempts was discontinued.

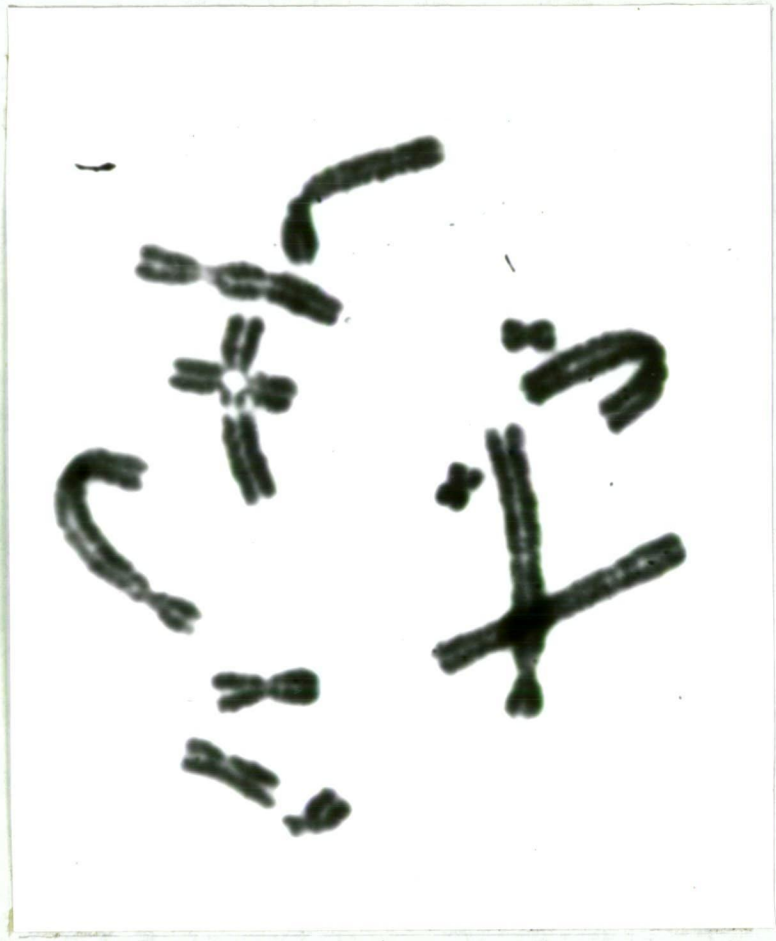


Photo 2: Chromatid interchange between X and chromosome 4 at the centromeric regions, and an acentric pair formed by a break in the heterochromatic region of X

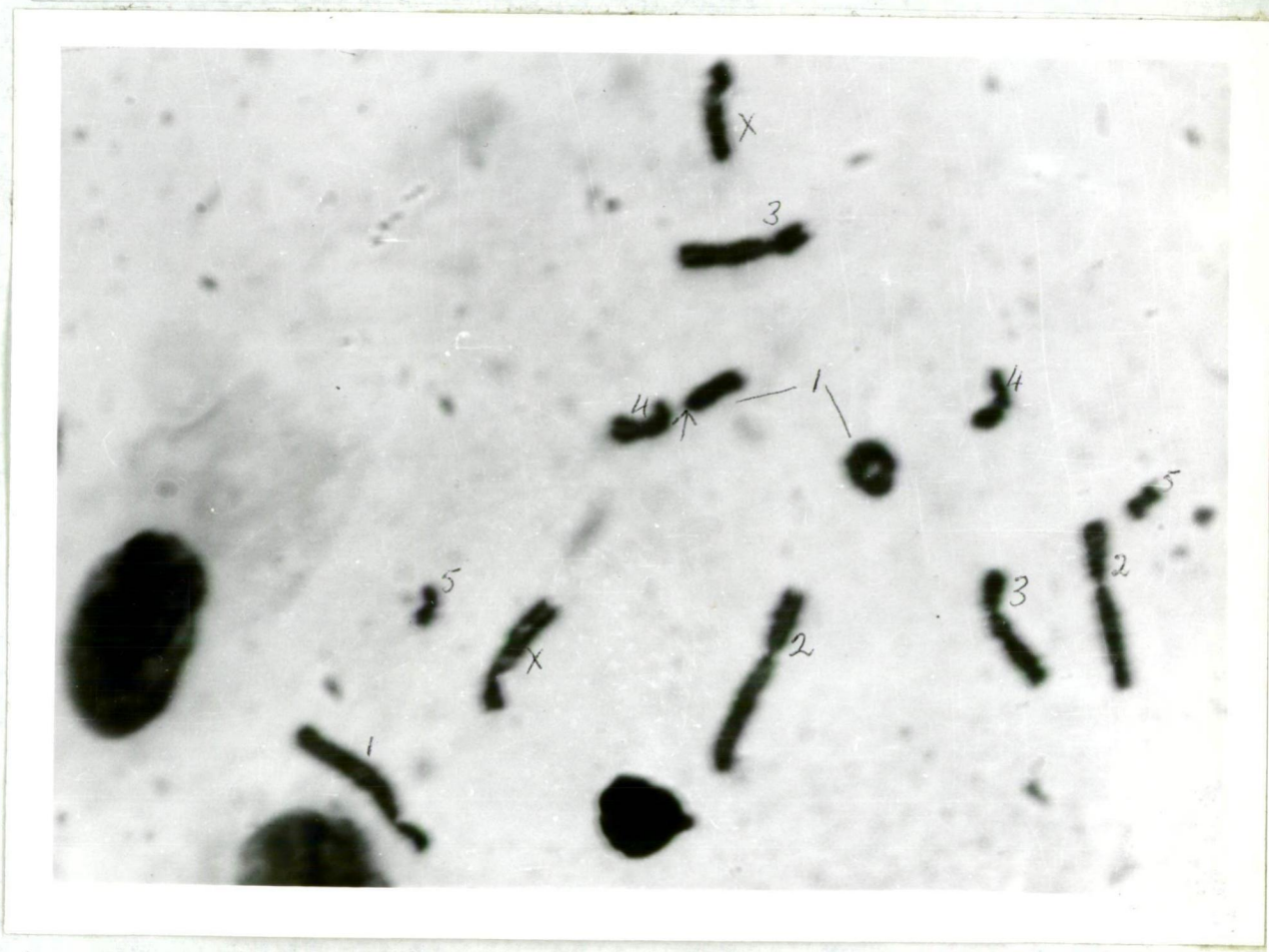


Photo 3: A ring and an acentric pair (arrow) formed from chromosome 1 by heliotrine treatment

Table V

Chromosome Damage by Heliotrine in Potoroo Leucocytes after 72 hours of Culture with Colchicine Treatment for a further 2 hours

Heliotrine Conc.	Chromatid deletions	ISO-CHROMATID DELETIONS NUpd	SU	NUp	NUd		TWO-HIT EVENTS Chromatid	Chromosome	Three-hit events
				5			0	0	0
0.05×10^{-4}M	7	4	0	1	4	9			
				11					
0.1×10^{-4}M	7	15	4	3	4	26	1	0	0
				1					
0.25×10^{-4}M	15	35	0	1	0	36	1	1	1 (chromatid)
				4					
0.5×10^{-4}M	40	26	2	0	2	30	8	6	1 (chromosome tricentric four-hit event)
				12					
1×10^{-4}M	66	52	0	4	8	64	8	0	4 (chromatid)
				2					
Control	1	3	0	0	2	5	0	0	0

Results given for 100 cells.

Legend for Table V

SU = sister union

NUp = non-union in proximal, i.e. centric portion

NUd = non-union in distal, i.e. acentric portion

NUpd = non-union in both centric and acentric

The chromosomal damage (table VI) was estimated as the number of breaks per nucleus: chromatid and iso-chromatid deletions were scored as one-hit events, while large interstitial deletions, dicentrics, rings, chromosome and chromatid interchanges were scored as two-hit events.

Table VI

Heliotrine Concentration	Number of hits/cell
$.05 \times 10^{-4}$M	.10
$.1 \times 10^{-4}$M	.29
$.25 \times 10^{-4}$M	.52
$.5 \times 10^{-4}$M	.95
1.0×10^{-4}M	1.52

For X-irradiated cultures stopped in the 72nd hour of the culture period the damage-dose relationship is shown in figure 3. The deviation from linearity in the dose-effect relation is possibly due to the greater mitotic delay with the higher doses. Experiments with potoroo leucocytes that were irradiated with 200r in the 60th hour of culture showed that virtually no cells reach metaphase till 6 hours after the Xray dose is given,

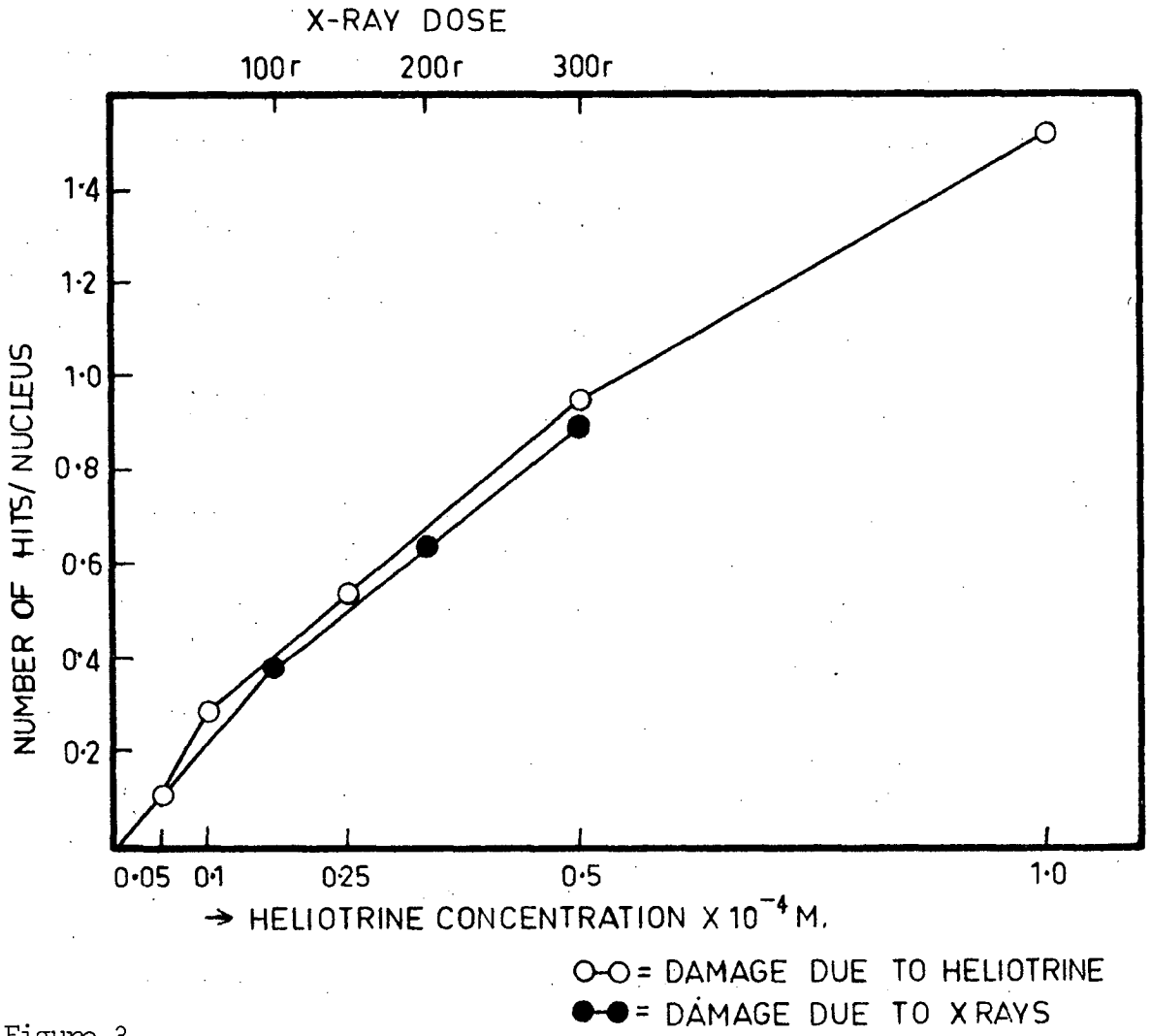


Figure 3.

Comparison of chromosomal damage produced by Xrays and heliotrine in potoroo leucocytes with colchicine treatment for a final two-hour period of culture (74 hours)

whereas parallel control cultures exhibited a good mitotic index anytime after 60th hour of culture. This increase in mitotic inhibition with larger doses of Xrays would prevent the more heavily damaged cells reaching metaphase at the time of cell fixation. The slopes of the dose/effect curves for X-irradiation and heliotrine treatment are similar (figure 3).

The dose/effect rate for the potoroo leucocytes is approximately .0033 breaks/cell/roentgen. This is somewhat lower than the figure of .0039 found by Bell and Baker[28] for human leucocytes and compares well with the value of .0031 obtained by Bender and Gooch[29] from experiments on human leucocyte cultures. The latter authors used only doses of 25r and 50r and with higher doses of the above experiments, the damage per roentgen could be expected to be somewhat higher.

In so far as a comparison can be made between the effects of Xrays delivered to the cells before the culture period and of heliotrine present in the medium for the whole of the period up to metaphase arrest, heliotrine concentration of 5×10^{-5}M produces about the same amount of final damage as 300r of Xrays delivered at 25r/minute.

The distribution of breaks between nuclei for both Xrays and heliotrine treatment was found to be non-Poissonian. These distributions are shown in figures 4 and 5. In all experiments, the variance:mean ratio proved to be significantly greater than unity, indicating considerable over-dispersion and an aggregation of damaging events. Figure 6 shows the variance:mean ratios calculated for the three doses of Xrays and for the different concentrations of heliotrine.

A non-random distribution of breaks between the individual chromosomes

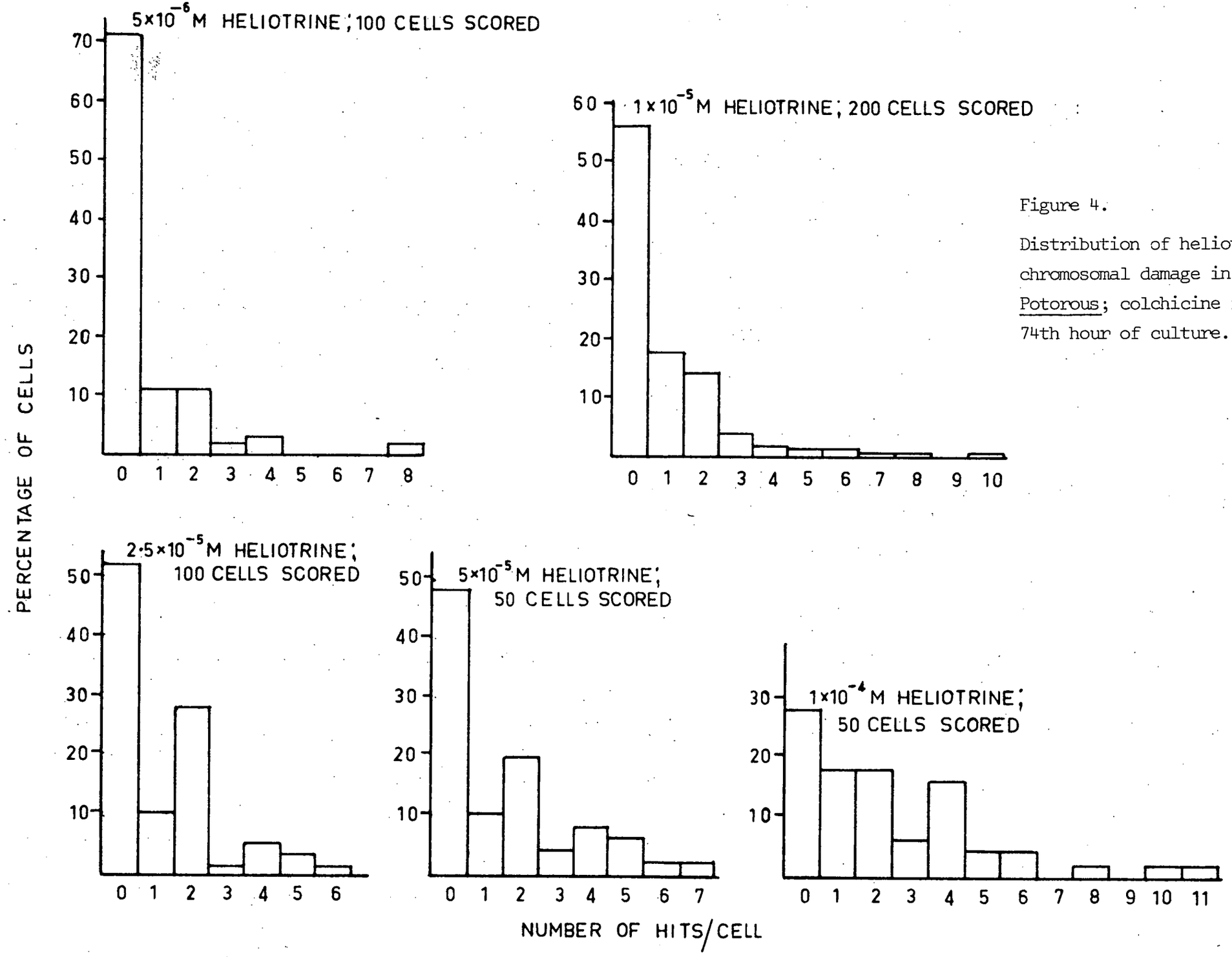


Figure 4.

Distribution of heliotrine-induced chromosomal damage in leucocytes of Potorous; colchicine from 72nd to 74th hour of culture.

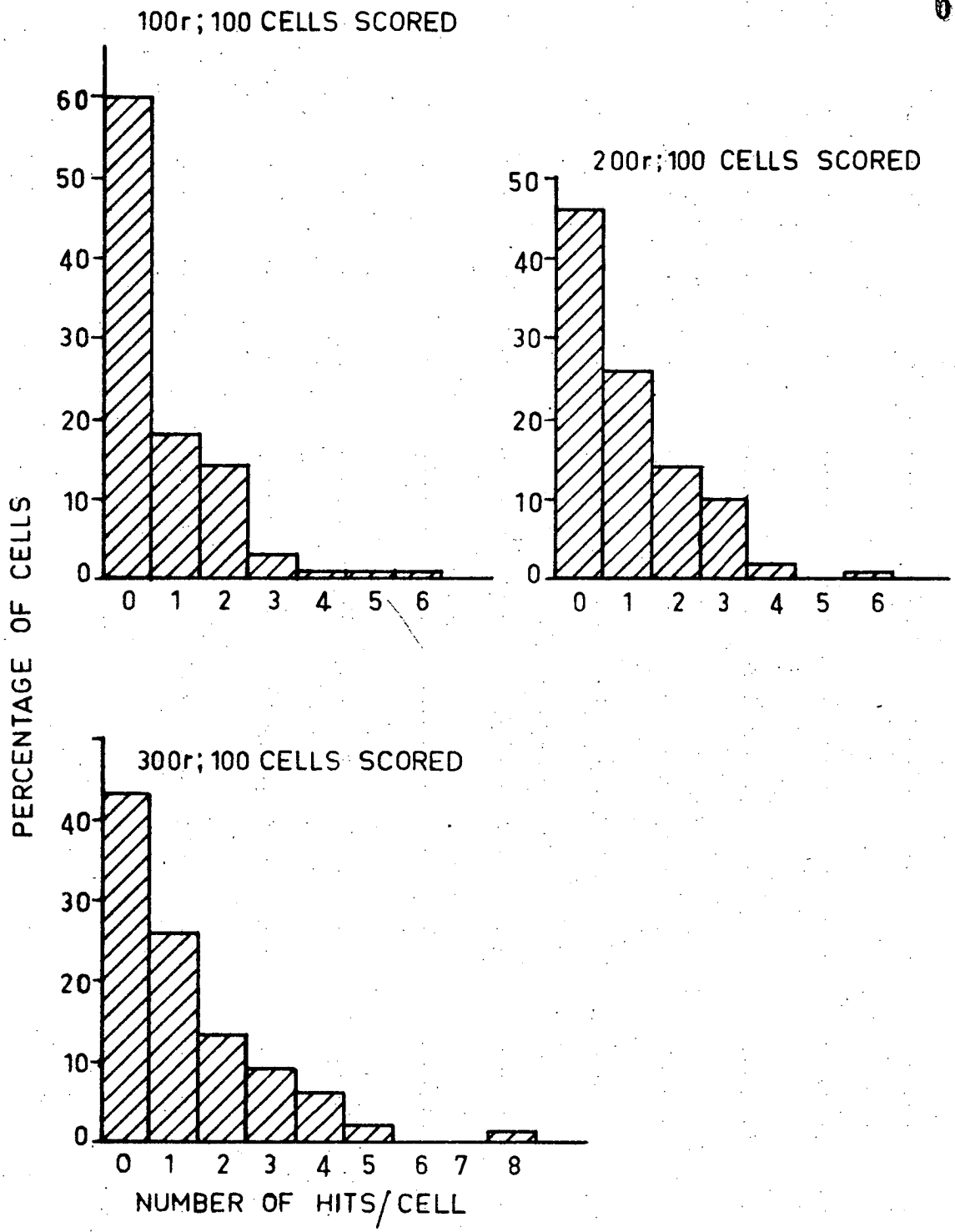


Figure 5.

Distribution of Xray induced chromosomal damage in potoroo leucocytes; colchicine from 72nd to 74th hour of culture; dose rate = 25r/minute.

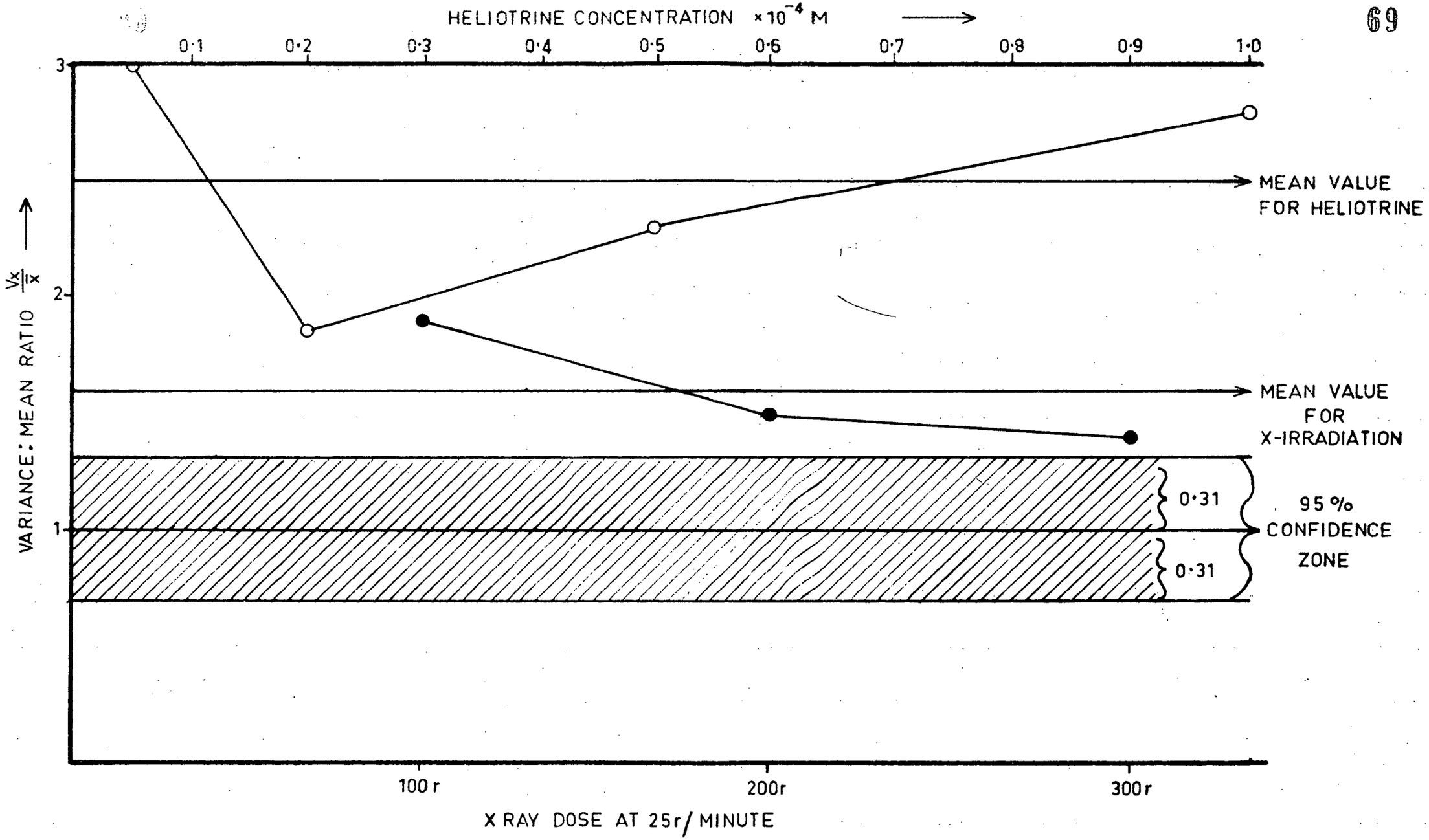


Figure 6. Ratio of observed variance at different doses of Xrays and heliotrine.
On the basis of random distribution of events a Poissonian distribution is expected with $\frac{\text{Variance O}}{\text{Variance E}} = 1$

of a nucleus has also been detected. The distribution of breaks induced by heliotrine is significantly different from that produced by Xrays (figure 7, table VII) and suggests some specificity of action either on initial breakage or subsequent process of repair.

Table VII

Distribution of breaks between chromosomes in relation to chromosome length

	Chromosome	Relative Length	Number of Hits	% of Total Hits	% Total Hits / Relative Length
	1	24.4	115	30.1	1.23
	2	22.7	97	25.4	1.12
X-rays	3	18.3	65	17.0	0.93
	4	10.4	23	6.0	0.58
	5	6.7	16	4.2	0.62
	X	17.3	65	17.3	1.00
Total	6	100.0	381	100.0	
	1	24.4	162	34.8	1.40
	2	22.7	96	20.6	0.91
Heliotrine	3	18.3	57	12.2	0.67
	4	10.4	40	8.6	0.82
	5	6.7	23	4.9	0.72
	X	17.3	88	18.7	1.08
Total	6	100.0	466	100.0	

Relative length is taken from the figures given by Shaw and Krooth[25]. For both Xrays and heliotrine, the discrepancy between the number of hits

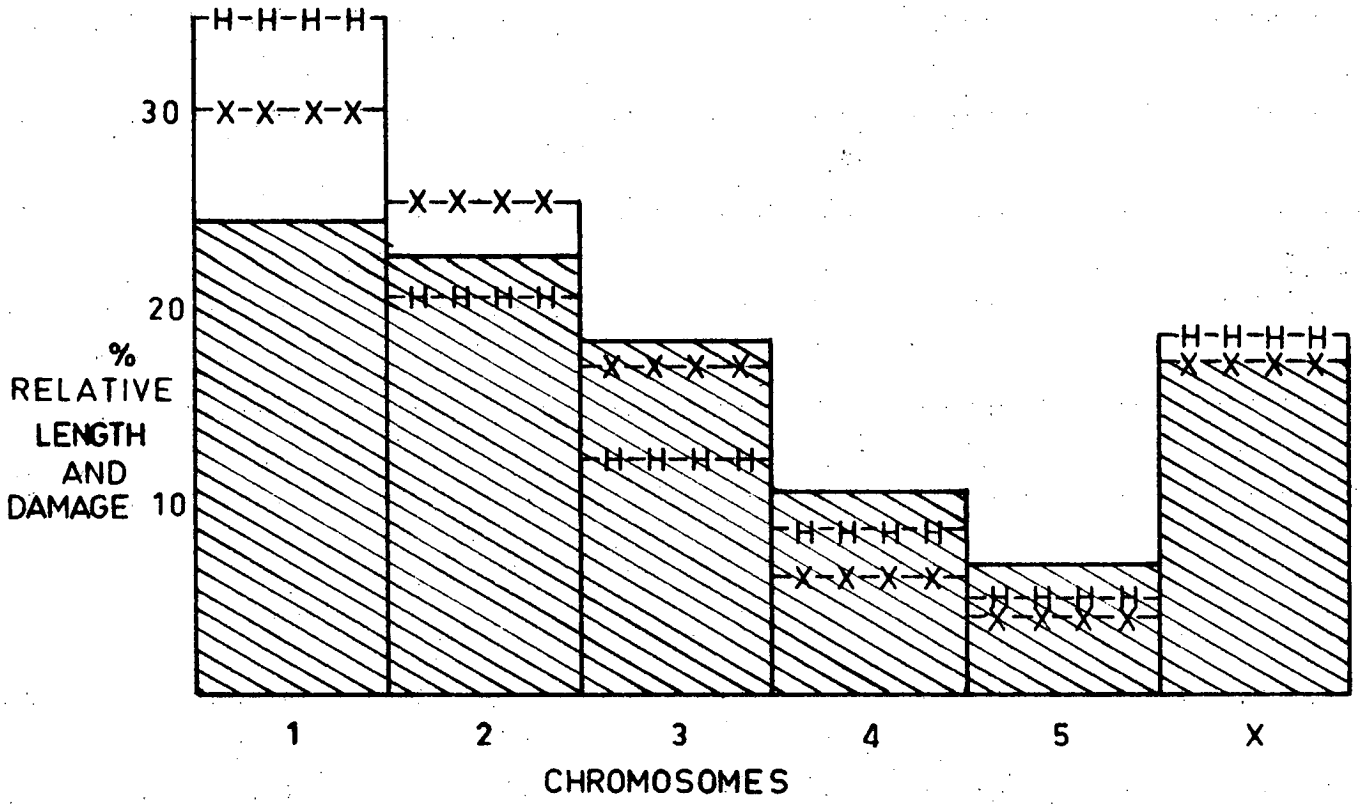


Figure 7. Distribution of damage between chromosomes of the potoroo complement produced by Xrays and heliotrine.

XXXX = relative damage produced by Xrays

HHHH = " " " " heliotrine

Shaded area = " size of chromosomes.

expected on the basis of a random distribution in terms of physical length and the number observed is statistically significant at less than the 1% level.

Both chromosome and chromatid aberrations were observed when heliotrine-treated leucocyte cultures were stopped in the 72nd hour. Hence it became important to examine only cells in first division after heliotrine treatment in order to determine the type of chromosome damage with certainty and to determine the stage of the cell cycle in which the alkaloid acts on the chromosomal complement.

There is some controversy in the literature as to the number of possible divisions in leucocyte cultures during the first 72 hours. At the time of setting up cultures, all the lymphocytes (the only leucocytes of the peripheral blood that are stimulated to divide by PHA) are in synchrony and start off in the G_1 phase. Buckton and Pike[30], using autoradiography for human leucocytes, found that after 72 hours many of the cells were in their second division. Sasaki and Norman[31] showed that in the 72nd hour of culture 70-80% of cells in metaphase are at least in their second division and that the cells proliferate with a generation time of 22 hours. However, when colcemid was added after the first 24 hours at a concentration of 3-4 µg/ml and the cells collected between 48 and 60th hour, then the authors found the cells to be in first division and the yield and quality of chromosome preparations for cytological examination were similar to those obtained with standard 72 hour cultures.

Some experimenters have scored from cultures stopped in the 72nd hour and presented the damage as though all the metaphases were in first divis-

ions after treatment[32]. Bender and Gooch[33] give data for chromosome damage from Xrays which they obtained by scoring metaphases from the 66th to 72nd hour of culture. All metaphases are treated as first divisions, "since previous experiments had clearly shown that in our laboratory virtually all mitoses occurring until about 80 hours after culture initiation were dividing for the first time in vitro."[33]

From data on the potoroo leucocytes (both the time of the appearance of first metaphases in culture and the subsequent mitotic index curve in figure 2), it did not seem likely that all the cells dividing in 72nd to 74th hour would be still in their first division. To ensure that only first division metaphases were scored, the procedure of Sasaki and Norman[31] was followed. Cultures containing three levels of heliotrine and controls were set up; after 24 hours, colcemid was added and the cultures were stopped in the 60th hour; the cells received hypotonic treatment and were fixed as before. The damage produced by three concentrations of heliotrine when only first divisions were scored is given in Table VIII.

If the iso-chromatid deletions for all heliotrine experiments are pooled together, then there are 74 closed or partially closed deletions (i.e. showing SU, NUp or NUd) and 230 open (i.e. NUpd). This gives a percentage of iso-chromatid deletions showing some form of sister-strand reunion as only 24%, while 76% of the iso-chromatid deletions are of the NUpd type. If this is compared with the iso-chromatid breaks produced by Xrays in the HPKI cells (potoroo kidney cells, experiments described in section H of this thesis) then out of 195 iso-chromatid deletions produced

Table VIII

Chromosome Damage by Heliotrine in Potoroo Leucocytes arrested in their first Division in vitro; Colcemid from 24th to 60th Hour of Culture

Heliotrine Conc.	Chromatid Deletions	ISO-CHROMATID DELETIONS NUpd	SU	NUp	NUd	TWO-HIT EVENTS Chromatid	Chromosome	Multiple Events	Cells Scored
1×10^{-4}M	36	43	11 (68)	8 (25)	6 (25)	3	2	3 chromatid triradial 1 eight-hit chromatid	100
0.5×10^{-4}M (average of 2 separate exps.)	28	34	3	4 (11)	4 (11)	5	1	2 chromatid triradial 2 four-hit chromatid 1 six-hit chromatid 1 eight-hit chromatid	100
0.1×10^{-4}M (average of 2 separate exps.)	18	21	2 (25)	1 (4)	1 (4)	1	0	0	100
Control (average of 2 exps.)	3	4	1 (5)	0 (1)	0 (1)	0	0	0	100

by irradiating the cells in S or G_2, 112 i.e., 57% showed some form of sister-strand reunion. Thus it seems highly likely that heliotrine-treatment keeps broken chromatid ends open, i.e. prevents them from rejoining. An alternate explanation is that a larger proportion of heliotrine-formed iso-chromatid breaks are not the result of a single event but of two separate events separated in time.

Another noticeable difference is the number of damaging events that involve the centromeres or other heterochromatin in the heliotrine-treated cultures compared with those that have only been X-irradiated. In X-irradiated cultures, a centromeric region might become involved in a translocation or a deletion but on an average of no more than once or twice in every 100 cells scored. For heliotrine-treated cultures, the centromeres or the heterochromatic segments of X are involved in over 20% of the chromosomal aberrations produced (Table IX).

Table IX

Comparison of damage produced by heliotrine in heterochromatic regions with the total damage

Heliotrine Conc.	Total No. of damaging events	% of events involving centromere or heterochromatic region of X	Cells scored
0.1×10^{-4}M	45	33%	100
0.5×10^{-4}M	113	26%	100
1.0×10^{-4}M	131	22%	100

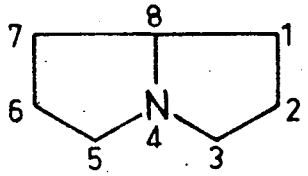
Potoroo leucocyte cultures: colcemid treatment from 24th to 60th hour; first divisions scored only.

Discussion

Heliotrine has a pyrrolizidine nucleus (I) in common with a number of other alkaloids. However, not all of these cause liver damage or produce mutations. Schoental[10] observed that hepatotoxic activity could be correlated with the presence of a double bond in the 1:2 position. Culvenor, Dann and Dick[34] have shown further that the biologically active pyrrolizidine alkaloids have an allyl ester group in position 1, (II) and have suggested that such alkaloids can act as biological alkylating agents. Heliotrine has the required structural features to act in this way (III), and moreover its mutagenic activity is associated with the intact molecule, since neither the acid nor basic fragments obtained on hydrolysis of the ester link proved to be mutagenic[15].

Alkylating agents are capable of reacting with deoxyribonucleic acid (DNA) to give phosphate triesters[35], or they can bring about direct alkylation of the purine or pyrimidine rings[36]. Since trialkyl phosphate esters are unstable and readily hydrolyzed in weakly alkaline solutions, the alkylated DNA would tend to disintegrate into smaller fragments[37].

As far as alkylation of the basic units of DNA is concerned, Brookes and Lawley[38] found that guanine is the most susceptible base to attack. Alkylation of guanine can produce three effects: (1) deletion of a base pair, i.e. guanine-cytosine (GC), (2) pairing of the alkylated guanine with thymine (T) instead of cytosine, so that after two replications of the DNA strand a GC to AT transition could occur, (3) direct interference with DNA replication due to alkylation of the DNA template. It is of interest that Somers and Hsu[39] believe that the centromeric regions in



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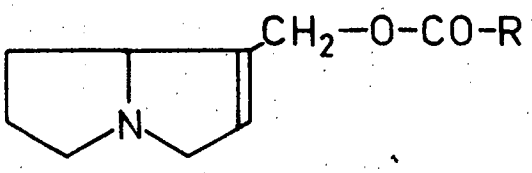

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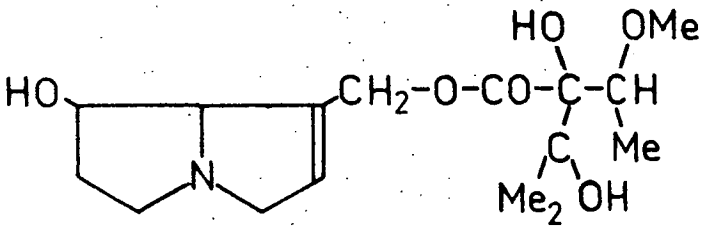

III HELIOTRINE

Chinese hamster cells have a high guanine-cytosine content. If this is so, and centromeres and the heterochromatic region of the X chromosome contain a higher proportion of GC pairs, then this could explain the preferential attack of these spots by heliotrine in the potoroo cells.

It is generally accepted that alkylating agents produce mutations and chromosomal aberrations by an alkylation of DNA, rather than of DNA precursors[40]. There appears to be general agreement that DNA is the most sensitive material to alkylation within the cell and is probably also the primary site of alkylation[41]. Evans and Scott[42] observed that breakage by alkylating agents occurs during S. Kihlman suggests that alkylation of DNA is possible only during S because at other stages of the mitotic cycle the DNA of the chromosomes is protected by other substances. It is also conceivable that although DNA may be alkylated at any stage, the alkylation results in chromosomal aberrations only in connection with DNA synthesis. Finally, there is the possibility that the direct cause of aberrations is not the alkylation of DNA, but the inhibition of DNA synthesis which the alkylation has produced. However, for alkylating agents, there appears to be no correlation between ability to inhibit DNA synthesis and ability to produce chromosomal aberrations[41].

Whether heliotrine or any of the other pyrrolizidine alkaloids which show mutagenic properties act primarily as alkylating agents of the DNA molecule itself still remains to be determined. Recent observations by Alderson and Clark[43] on Aspergillus nidulans indicate unexpected differences in specificity of chemical mutagenesis between alkylating agents such as diethyl sulphate and ethyl methane sulphonate on the one hand, and between

mutagenic alkaloids such as heliotrine and lasiocarpine on the other.

Both X-irradiation and heliotrine treatment produce a non-random distribution of breaks which, however, is more pronounced in the case of heliotrine (fig. 4). Overdispersion following X-irradiation has been discussed by Jackson and Barber[44] in relation to the hypothesis of a cumulative action of events subsequent to the absorption of radiant energy. In the case of a chemical mutagen, physiological differences between the cells making up the population, along with the possibility that the entrance of a first group of heliotrine molecules into a cell might influence the path followed by other succeeding molecules, could provide a hypothesis to account for the non-Poissonian nature of the distribution.

The non-random distribution between individual chromosomes of Vicia faba after X-irradiation has been observed by Roger and Michaelis[45], while Evans and Bigger[46] found there was an association between the distribution of Xray-induced aberrations and the distribution of heterochromatin, there being a tendency for more aberrations to occur in or near heterochromatic regions. As shown by Kihlman and Eriksson[47], an even more marked deviation from the expected Poisson distribution of aberrations is obtained after treatments with certain chemical agents. McLeish[48] found in Vicia faba particular regions of chromosomes more susceptible to breakage by chemical treatment.

Several authors[46,48,49,50,51] have pointed out that an important factor influencing the distribution of aberrations is the organization of the interphase nucleus. There is probably a connection between the

tendency of the aberrations to be concentrated in the heterochromatin and the fact that heterochromatic segments tend to fuse in chromocentres in the interphase nucleus. Thus damaged chromosomes can interract to form aberrations of interchange type if they are in close association. (Chromatid and iso-chromatid breaks should be regarded as aberrations of exchange type according to Revell[53].) The presence of the nucleolus could act as a physical barrier to all types of chromosome-breaking agents. In cases where chromosome breakage is the result of an inhibition of synthesis of chromosome material, aberrations would occur more frequently in regions where synthesis took place at the time of treatment. As a rule, heterochromatic regions are late in synthesising their DNA[52].

In the potoroo experiments outlined above, the distribution of breaks between individual chromosomes, after X-irradiation and heliotrine-treatment, appear to be non-random. Following X-irradiation, the large autosomes 1 and 2 are found to have a greater proportion of damage than would be expected on the basis of their relative length. Damage to the small chromosomes 4 and 5 is less than the expected value, whereas medium-sized chromosomes, 3 and X, received a number of damaging events closer to the expected value (on the basis of relative chromosome lengths). Since damage in the large chromosomes is particularly easy to detect, it is possible that the observed non-random distribution of breaks between the chromosomal elements is in part artificial, due to differences in the efficiency in scoring. The distribution of breaks induced by heliotrine is significantly different from Xray damage, and there is a predominence of damaging events affecting the centromeres and the heterochromatic

region of the X chromosome. This suggests some specificity of action of the alkaloid on initial breakage or on subsequent process of repair.

The production of both chromosome and chromatid aberrations in Potorous leucocytes by heliotrine is not in harmony with data available for plant material. Avanzi[18,19] pointed out that in Allium and Vicia root tips, pyrrolizidine alkaloids produce only chromatid-type aberrations, even after 3 or 4 days following alkaloid treatment when derived chromosome aberrations might be expected.

Davidson[54] noted in Vicia the inability of azaserine to induce breaks in unsplit chromosomes. Aberrations are exclusively of the chromatid type, with breakage localised in heterochromatic regions. He concluded that in plant material, radiomimetic chemicals fail to induce true chromosomal aberrations such as rings and dicentrics.

Somers and Hsu[39] found, however, that hydroxylamine (HA) produces large numbers of both chromosome and chromatid aberrations in cultures of Chinese hamster cells. It also produces constrictions and despiralization of chromosomes. The exchange type of aberration proved to be frequent and a high percentage of the damage was localized in the centromeric regions of the chromosomes. HA is thought to react primarily with the cytosine moiety of DNA[40], but these authors suggests that the chromosomal aberrations induced by HA and its derivatives might be the result of main-chain scission of DNA rather than of a reaction with the cytosine of DNA.

Another radiomimetic substance which has been shown to produce chromosome and chromatid type of aberrations is 8-ethoxycaffeine (EOC).

Exchanges and SU-isochromatid breaks occur with the same relative frequency as after X-irradiation which indicates that rejoining is not inhibited[47]. Kihlman[40] believes that chromosomal DNA or DNA-protein complex probably suffers a physico-chemical attack by EOC, although the dependence of the effect on oxidative phosphorylation shows that enzymatic reactions are also involved. EOC induces mainly iso-locus breaks and has a non-delayed effect when used in concentrations of 2 to 10 x 10^{-3}M [47]. Scott and Evans[55] (1964) found that cells are arrested by EOC mainly in the G_2 stage of interphase, which is prolonged to about twice its normal duration.

Conclusion

Heliotrine has a strong inhibitory action on the mitotic index of marsupial cells in tissue culture. Below 1 x 10^{-6}M, the effects of the alkaloid are very slight on the rate of cell division. At concentrations in excess of 5 x 10^{-6}M, heliotrine produces breakage of potoroo chromosomes. In this effect it appears to differ from the majority of radiomimetic substances: unlike other alkylating agents that cause only chromatid breaks by an action during chromosome replication (S), heliotrine treatments show in addition chromosome breakage (1 to 2%) for concentrations of 5 x 10^{-5}M.

That these are in fact chromosome breaks caused by the heliotrine treatment rather than aberrant chromosomal damage resulting from the entry of cells with broken chromatids into a second division is established by the following observations:

1. Control cultures without heliotrine show only 1 to 2 cells in division per 10,000 cells at the 24th hour of the culture period, and about 1% of cells in division at the 48th hour; thus it would be possible to obtain some cells in their second division at the 60th hour.

2. Heliotrine treatments of 5×10^{-5}M, however, produce a mitotic delay of at least 10 hours, and no divisions are observed at the 24th hour of culture. At the 48th hour, the number of dividing cells observed is only 0.2%. It is thus most improbable that any cells in these cultures could pass through two mitotic cycles before the 60th hour.

3. The addition of colcemid to these heliotrine-treated cultures at the 24th hour, i.e. before any cells came up for division for the first time, ensured that no cell was in second division at the 60th hour when the scoring was carried out.

4. Those cells which showed apparent chromosome breaks, such as rings and dicentrics, invariably carried all the expected deleted fragments; this observation strongly supports the conclusion that these aberrations are in fact real chromosome breaks.

Autoradiographic studies in conjunction with heliotrine experiments would be necessary to confirm the conclusion that chromosome aberrations observed are in nuclei of cells in their first division. By the use of higher concentrations, it might be possible to induce a larger proportion of chromosome aberrations (e.g. EOC has to be used in concentration of 2 to 10×10^{-3}M to produce non-delayed effects). The higher concentrations of the alkaloid would have to be introduced for short periods in G_1, since a concentration of 2×10^{-4}M for the total period of culture suppresses

the mitotic index of potoroo cells to such an extent that none reach metaphase in the allotted time.

Heliotrine resembles the action of hydroxylamine more than that of 8-ethoxycaffeine. Like HA, heliotrine produces secondary constrictions and despiralization of chromosomal material. Both of these substances cause a high percentage of localized damage in centromeric regions of the chromosomes, and both can act by a direct attack on the DNA molecule; moreover, they are both capable of reacting with base moieties of DNA - hydroxylamine with cytosine and heliotrine with guanine (in consequence their attack would occur in the same region of the chromosome, since the purine guanine pairs with the pyrimidine cytosine). In both cases, part of the damage caused is considered to be due to the scission of the DNA chain which leads to visible cytological gaps in the chromosomal structure.

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F. Chemical Protection against X-irradiation by a new reducing Agent, 1,4-Dithiothreitol (DTT) in marsupial Leucocytes in Culture.

Introduction

Chemical changes produced by radiations such as Xrays are brought about by two distinct mechanisms[1]: 1) by direct action when the molecule undergoing change becomes ionised or suffers cleavage into radicals by passage of an electron, other atomic particle or electromagnetic radiation of suitable wave length, and 2) by indirect action, in which the molecule in question does not absorb the energy directly but receives it by transfer from another excited molecule. In biological systems, water is the invariable solvent and the activation of water molecules leads to the formation of free radicals carrying an unpaired electrom which makes them extremely reactive. Most free radicals persist for no more than 10^{-5} sec., and for all practical purposes primary changes can be regarded as instantaneous[1].

The free radicals are formed when the ionising particle (such as an electron from a β ray) in its passage through water ejects an electron to produce a positive ion[2]:

$$H_2O \xrightarrow{\text{radiation}} H_2O^+ + e \qquad (1)$$

The electron is then captured by another water molecule to give a negative ion:

$$H_2O + e \rightarrow H_2O^- \qquad (2)$$

H_2O^+ and H_2O^- are different from stable ions and decompose almost immediately:

$$H_2O^+ \rightarrow H^+ + OH^\cdot \quad (3)$$

$$H_2O^- \rightarrow OH^- + H^\cdot \quad (4)$$

to give two free radicals and two stable ions H^+ and OH^- which recombine to give water. Indirect action depends on the reactions with cell constituents of the $H^\cdot$ and $OH^\cdot$ radicals formed in water. Some of the free radicals are removed by recombination with themselves:

$$H^\cdot + OH^\cdot \rightarrow H_2O \quad (5)$$

$$H^\cdot + H^\cdot \rightarrow H_2 \quad (6)$$

$$OH^\cdot + OH^\cdot \rightarrow H_2O_2 \quad (7)$$

The hydrogen peroxide so formed is a strong oxidising agent which can also attack many substances within the living cell and which contributes to the harmful effects of activated water[2]. If oxygen is present, further reactions can take place:

$$O_2 \xrightarrow{\text{radiation}} O^\cdot + O^\cdot \quad (8)$$

$$O_2 + H^\cdot \rightarrow O_2H^\cdot \quad (9)$$

$$O_2 + 2OH^\cdot \rightarrow 2O_2H^\cdot \quad (10)$$

Free radicals will interact with a great variety of biological metabolites. Added substances will compete for the radicals and thus reduce the damage received by cell constituents. Added proteins protect those naturally present since they react approximately equally with free radicals formed in water[3]. The damage produced by direct action (i.e. when radiation damage is inflicted on dry macromolecules) can also be lessened by addition of chemical protectors[4].

Chemical protection in the latter case has been divided by Bacq and

Alexander[1] into the following:-

1. Energy transfer type: the energy deposited in one molecule exerts its chemical effect at another place by the addition of substances which act as "receptors" of energy.

2. Repair of damaged molecule: this mode of action relies on the fact that the first chemical change need not be irreversible. There may be a short time during which the molecule can react with a protector so that it is restored to its original state.

3. Protection of a complex organism as a whole by the presence of a protector with a pharmacological action which changes the response of the whole organism to radiation.

When the damaging reaction occurs indirectly, i.e. when it is produced by free radicals in aqueous solutions, then protection can take place if substances are introduced that can compete for these radicals which otherwise would attack "target" molecules within the cell. This protection by competition was discovered by Dale[5], who found that certain substances protected dilute solutions of enzymes (Sulphur-containing compounds were found to be exceptionally effective). On the other hand, protection may occur not as a result of competition for damaging radicals but by promotion of repair; qualitatively, the same effect would be obtained if the added substance had combined not with the attacking radical but with the damaged molecule and restored it to its original state[1].

Protective compounds can be classified into 4 main groups[6]:-

1) Sulfhydryl compounds [compounds with -SH groups such as cysteine,

BAL(2-3 mercaptopropanol) and mercaptocarboxylic acid] with distinct, almost immediate, effects even at 0.001M concentrations without prior incubation in the medium surrounding the living cells. The substance DTT , which is to be discussed in this section, belongs to this group.

2) Strong reducing agents (e.g. $Na_2S_2O_4$, sodium hydrosulphite), highly effective without prior incubation.

3) Alcohols (ethyl alcohol, glycerine, glycols) which require higher concentrations and are much more effective after organisms are incubated for 1/2 hour in the medium surrounding them.

4) Salts of carboxylic acids, effective at very low concentrations, e.g. 0.0001M, but requiring pre-incubation.

Whereas actions of two substances of the same group have been shown to be additive, those of groups 1, 2 and 3 tried in combination act synergistically; their effects evidently are different in kind or result from action at different sites[6]. Group 1 presumably protects by offering alternative SH groups in addition to those occurring naturally in many proteins, while group 2 protects directly through its reducing activity. The related groups 3 and 4, requiring incubation, apparently require metabolic changes before they can exercise effective protection. Stapleton and Billen have found that respiratory inhibitors such as cyanide inhibit these changes and thus block the development of the protective property[6].

It is generally accepted that protective compounds have to be present in the organism during irradiation, and it is generally considered that in <u>vivo</u> conditions the penetration of the protector from the surrounding

medium into the cells, is a prerequisite for protection[14]. Contrary to all other work with protective compounds is the finding by Künkel et al.[15] that cysteine protects the irradiated dormant dormouse (Glis glis) when it is injected after irradiation and just before the animal is aroused from hibernation by warming to room temperature.

The most effective and useful group of protective chemicals are the sulfhydryl compounds[7]. These substances have been exhaustively investigated ever since the discovery by Patt and his coworkers[8] that cysteine could reduce the number of deaths from radiation treatments in mammals. They have been shown to protect organic macromolecules in cell-free solutions[9], bacteria[10], plants[11,12] and animals[7,8,13] against irradiation damage. In root-tips of Vicia faba Riley[18] obtained optimum protection with 2×10^{-3}M of BAL. Wolff[11], also working with Vicia, found that the protection afforded by BAL against X-irradiation resulted in a marked reduction in the number of two-hit aberrations. There is some difference, however, in the protection afforded to different species according to the time of administration of these -SH compounds. A concentration of 1×10^{-2}M of cysteine was found to give optimal protection in yeast cells 10 minutes after the application of the chemical[16]. In mice, cysteamine affords optimal protection 10 minutes after administration, but in rats, maximum protection occurs only 45 minutes post administration[17].

Sulfhydryl compounds might be effective through several different mechanisms. It has been suggested[18] that they may simply act as reducing agents by removing available oxygen, since none of these chemicals (such

as BAL and cysteine) give increased protection over that produced by simple oxygen removal, by which the irradiation damage is halved[19]. In the absence of oxygen, sulfhydryl compounds and reducing agents lose most of their ability to protect against radiation[12]. Moreover, cysteine has only a slight protective action against inhibition of growth induced by α particles, which are almost equally effective in the absence of oxygen as in its presence[1].

These -SH containing compounds might also protect by acting as scavengers of free radicals[20] produced by the chain reactions, thus protecting by competition sensitive -SH sites in proteins. For instance, sulfhydryl compounds could react with hydroxyl radicals to form much more stable and less reactive sulphur-containing radicals:

$$R\text{-}SH + OH^{\cdot} \rightarrow RS^{\cdot} + H_2O \qquad (11)$$

It is possible that these compounds may act by forming complexes with cellular metabolites. Eldjarn and Pihl[22] have suggested that substances such as cysteine and BAL may protect by reacting with other -SH or -S-S- groups in proteins of animal tissues. The normal -S-S- bonds have been found to be more sensitive to irradiation than the rest of the molecule, and the formation of a mixed disulphide by the addition of the radioprotector should make the compound more resistant to indirect, i.e. free radical action, as well as direct action; subsequent restoration of the -S-S- protein groups would occur[1].

Another more general way in which certain chemicals can apparently decrease aberration yield is simply by raising the pH of the cells.

Marshak[21] showed that ammonium hydroxide treatment lowered significantly the frequency of radiation-induced aberrations in Vicia. It is yet uncertain whether the differences are the result of decreased breakage or increased rejoining.

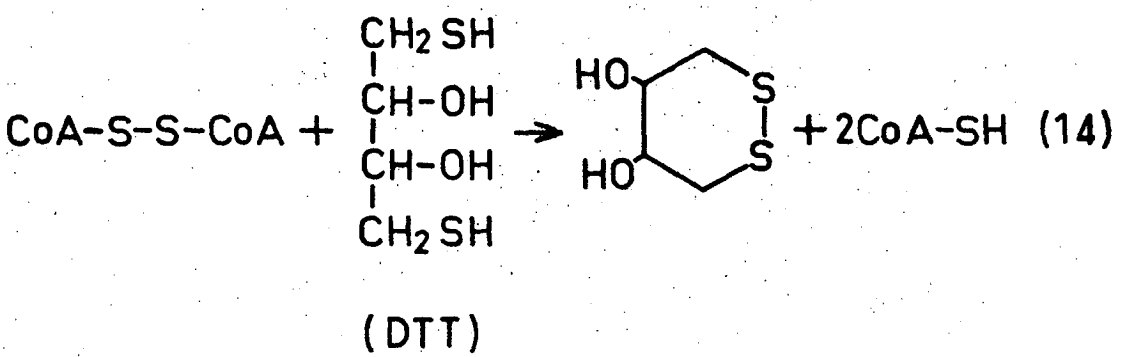
Sulfhydryl compounds may also repair damage, e.g. in the case of a coenzyme A molecule (Co A-SH) which has been inactivated by conversion through a free radical oxidation to a disulphide[23]:

$$CoA\text{-}S\text{-}S\text{-}CoA + RSH \rightarrow CoA\text{-}SH + R\text{-}SS\text{-}CoA \qquad (12)$$

$$R\text{-}SS\text{-}CoA + RSH \rightarrow R\text{-}SS\text{-}R + CoA\text{-}SH \qquad (13)$$

The high reactivity of sulfhydryl compounds makes it all the more difficult to determine the mode of their action. Graevsky et al.[24] suggest that aminothiols act as protectors because of their ability to increase the content of endogenous sulfhydryl compounds. They postulate that the radiosensitivity of a biological tissue depends on its content of sulfhydryl compounds, and have shown that an increase in the concentration of sulfhydryl groups occurs in the tissues both under anoxic conditions and in the presence of various chemical protectors. They point out that the inactivity of aminothiols at low temperatures supports their contention that it is not the aminothiols themselves which protect against radiation but endogenous sulfhydryl compounds catalysed by the aminothiols[24].

Recently (1963), dithiothreitol (DTT) was put forward by Cleland[23] as a new protective agent for SH groups against oxidation. Since the molecule has two sulfhydryl groups, its effectiveness is doubled, and should be further enhanced in the repair mechanism mentioned above (equations 12 and 13), since the disulphide formed is a sterically-favoured



(DTT)

cyclic one (equation 14). This increases its effectiveness, and gives it a low redox potential[23]: it is capable of maintaining monothiols in the reduced state and reducing dithiols quantitatively[23]. Since DTT is a highly water-soluble solid with a very low vapour pressure and little tendency to be oxidized by air, it should prove superior in these respects to the thiols already in use, some of which are sparingly soluble or have an objectionable smell[23]. No data have so far been reported on DTT as a protective agent against irradiation damage, for which it would appear to be particularly well adapted. The following experiments were carried out to see to what extent DTT minimised chromosome breakage in leucocyte cultures of the marsupial, Potorous tridactylus.

Materials and Methods

Blood was obtained by heart puncture from a potoroo anaesthetised by injection with Nembutal (sodium penta barbitone, 60 mg/ml; 1 ml/4lbs body weight). The blood was collected in a heparinized syringe and placed in a flask with a few drops of heparin (2 drops of heparin per 10 ml of blood). The blood was fully oxygenated by blowing air through it for 30 minutes with continuous stirring with a magnetic stirrer. Sterile apparatus and aseptic techniques were used throughout the experiment.

Five ml aliquots of blood were placed in sterile centrifuge tubes half of which received 0.5 ml of DTT (i.e. 0.1 mg of DTT/1 ml whole blood) per 5 ml of whole blood one hour before irradiation. Blood was irradiated with 200r at 100r/minute [H.V.L. 0.5 mm Cu; 235 KV Peak]. Blood for control

cultures was left at room temperature. All blood samples were then mixed with a few drops of phytohaemagglutinin, PHA (Wellcome), and after 10 minutes centrifuged for 5 minutes at 100g. The supernatant plasma with suspended leucocytes was removed to fresh tubes and the leucocytes precipitated by centrifuging for 7 minutes at 150 g. The supernatant was discarded and the cells were washed twice with 5 ml of complete medium to remove all traces of DTT and autologous serum. One half ml of complete medium was added to each batch of washed leucocytes from 5 ml blood; the cells were dispersed by gentle pipetting and placed in culture flasks containing 8 ml of complete medium.

The culture medium consisted of Parker's 199 medium, 12% foetal calf serum (both from Australian Commonwealth Serum Laboratories) and 2% PHA. DTT solution was made by dissolving 50mg of dithiothreitol (Cleland's reagent, Calbiochem) in 50 ml of Hank's balanced salt solution (B.S.S. also from Aust. Commonwealth Serum Labs.) and sterilized by filtration.

The cultures were incubated for 72 hours at 37°C, then for a further 2 hours with added Colchicine (Ciba) to give a final concentration of 0.004% in order to arrest cells in mitosis. The cultures were then centrifuged and the supernatant removed except for 1/2 ml of medium in each tube; one ml of warm distilled water (37°C) was added and the cells dispersed quickly by gentle pipetting. The cells in this hypotonic solution were incubated at 37°C in a water bath for 10 minutes. One and a helf ml of freshly prepared fixative (1:3 of glacial acetic acid and absolute ethanol) were added to each tube and the cell suspensions were left at room temperature for half an hour. The cells were centrifuged

down, the supernatant removed and the cells resuspended in 1/2 ml of freshly prepared fixative. After 30 minutes, slides were made by placing a few drops of the cell suspension on a slide and adding two to three drops of 60% acetic acid. The slides were dried on a warming plate at 60°C, fixed by immersing in absolute methanol and then stained with Leishman's stain.

Results

All cultures gave good mitotic indices and chromosomal damage was scored as follows: chromatid and isochromatid deletions were taken as one-hit events; chromosomal translocations such as conjugation of acentric pair with another chromosome, and the formation of rings and dicentrics, were scored as two-hit events.

The low chromosome numbers and distinct morphology of the potoroo chromosomes enable an accurate estimation of damage at metaphase, and translocations involving two or more chromosomes can be recognised without ambiguity. Since the whole cell population was in the pre-DNA synthetic phase, i.e. in G1 at time of irradiation, the whole of the inflicted damage would be expected to be of the chromosomal and not the chromatid type. The results of the experiments are shown in Table I.

The presence of DIT decreases the radiation-induced damage of all types of aberrations. The iso-chromatid deletions are reduced by 20% and the two-hit events such as chromosome translocations by as much as 68%. If the damage is scored as the number of hits per cell, then the presence of DIT during irradiation reduces significantly the total damage which is

Table I

	Chromatid Deletion %	ISO-CHROMATID DELETIONS NUpd%	SU%	NUp%	NUd%	TWO-HIT EVENTS Chromatid	Chromosome	Cells scored
200r @ 100r/min								
DIT present								
Experiment I 1st hundred	5	54	0 (66)	12 (1)	11	0	5	100
2nd hundred	2	51	1 (63)	12 (3)	8	0	7	100
Experiment II	2	51	1 (62)	11 (7)	3	0	10	100
Average for above	3	52	1 (64)	12 (4)	7	0	7	300
200r @ 100r/min DIT absent								
Experiment I	10	62	2 (79)	17 (8)	7	0	16 + 1 tricentric	100
Experiment II	8	54	4 (70)	16 (6)	6	0	26	100
Average for above	9	58	3 (75)	17 (7)	7	0	22	200
Control	2	8	0 (10)	2 (1)	1	0	0	100

Legend for Table I

Leucocytes of Potorous from fully oxygenated blood, 72 hours in culture, colcemid treatment for the last 2 hours of culture period.

NUpd = non union in both centric and non-centric portions
SU = sister union
NUp = non-union in centric portion
NUd = non-union in acentric portion

only half that produced in the absence of this radioprotector (Table II).

Table II

	Number of hits/cell	No. of hits/cell allowing for spontaneous aberration	Cells scored
200r @ 100r/min DTT absent	2.18	2.06	200
200r @ 100r/min DTT present	1.11	0.99	300

Discussion

These experiments show beyond doubt that dithiothreitol is a powerful chemical protector for living cells against ionising radiations, and that it reduces significantly all types of chromosomal damage. Since from our previous experiments (section E), it would seem that a number of potoroo lymphocytes arrested in metaphase after 72 hours in culture are not in their first division, it would be valuable to score cells in their first division only. This would give a clearer picture of the exact specificity of action of this protective thiol on different types of aberrations.

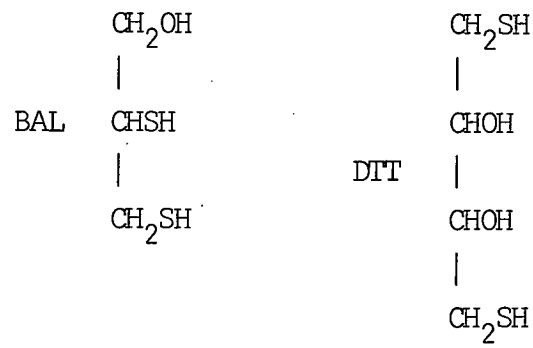
Wolff and Luippold[25] found that by changing the time between two dose fractions of Xrays, an interval of time can be found above which the fractions act as though they had been delivered independently. They found that below this time interval the breaks induced by the first fraction were still able to react with those produced by the second dose to give an increase in the number of two-hit events. Working with Vicia faba, Wolff[11,26] found that BAL, besides decreasing the number of aberrations, also shortened this

interval of time, thus decreasing the period during which the breaks remained capable of reacting with other breaks. It is due to this property of BAL that there is a decrease in the number of two-hit events. In our experiments, the most significant decrease in the observed aberrations is in the two-hit type of chromosomal damage. Thus it is possible that, like BAL, DTT acts by shortening the time that chromosome breaks remain open.

Work by Wolff and Atwood[27] led to the conclusion that increase of dose rate produces longer periods during which breaks remain open. This they thought might be the result of the destruction of some metabolic activity which was somehow protected if BAL was present. Further experiments by Wolff and Luippold[25,28] and by Cohn[29,30,31] showed that respiratory inhibitors and low temperatures had the opposite effect to that of BAL and anoxic conditions. Respiratory inhibitors extended the time during which breaks remain open, while BAL and anoxia reduced this open period. They concluded that active oxidative metabolism, which could be somehow protected by the presence of BAL, was required to produce healing of broken ends.

The chemical formulae of BAL and DTT are shown in figure I. The alcohol from which DTT is derived (by interchange of sulphur for oxygen) has an extra carbon atom as compared to the parent alcohol of BAL, and the two -SH groups of DTT are separated by two carbon atoms and not adjacent as in BAL. Because of the similarity of their chemical structure, it is likely that these two substances have a similar mechanism for their radioprotective action, but DTT for the reasons mentioned above has a

Figure I



lower redox potential than BAL[23,46].

The -SH groups that exist in the cell both in peptides and free amino acids and as an integral part of protein play an important role in the processes of cell division and growth[32]. The -SH groups in the protein molecules of a large number of enzymes (and the -SH group of coenzyme A) are also known to be essential for their activity. These enzymes were found to be readily inhibited because of the ease with which they could be oxidised by ionising radiations[33]. Thiol compounds such as glutathione and BAL are also readily oxidised by X, β and γ rays[32].

The oxidation of the sulfhydryl groups of metabolites could occur as follows[34] -

$$2RSH + 2OH^{\cdot} \rightleftarrows RS\text{-}SR + 2H_2O \qquad (15)$$

$$2RSH + 2O_2H^{\cdot} \rightleftarrows RS\text{-}SR + 2H_2O_2 \qquad (16)$$

$$2RSH + 2O^{\cdot} \rightleftarrows RS\text{-}SR + 2H_2O_2 \qquad (17)$$

$$2RSH + H_2O_2 \rightleftarrows RS\text{-}SR + 2H_2O \qquad (18)$$

The H_2O_2 produced in equations (16) and (17) would itself act as an oxidising agent as in (18). If the Xray dose was sufficiently low, Barron et al.[33] found that enzyme reactivation occurred on the addition of glut-

athione, which converted the disulphide groups back into the sulfhydryl stage. The irreversible inhibition which occurs when the dose of Xrays is increased is attributed to protein denaturation. From the above evidence, it seems likely that the presence of BAL and DTT could protect to some extent the -SH containing compounds within the cell by increasing the number of -SH groups available and thus diminish the indirect action of the products of irradiated water.

There is still no single theory which explains satisfactorily the prophylactic action of sulfhydryl compounds. Recently, Brown[34] (1967) has advanced a unifying theory for radioprotectors which are aminothiols. The most effective of these compounds are those closely related to cysteamine, $HS-CH_2-CH_2-NH_2$, or those that are readily metabolised to this type of compound. Brown points to the existence of experimental evidence that DNA is one of the sites of the primary radiation damage in cells. The nature of this damage appears to be single strand breakage followed by deletions and chemical alterations of the bases together with dissociation of histones.

In order to survive, a cell must successfully replicate a set of nearly normal DNA. Three processes, damage, repair and replication become the governing factors: as long as the rate of repair is greater than the rates of damage and replication, the cell should survive. The importance of relative rates is borne out by experiments which have shown the dependence of lethal radiation on the dose rate[35]. Survival will be favoured by decreasing both the rate of damage and the rate of replication. If a substance becomes bound to DNA, the DNA helix becomes more stable and both

of these requirements will be fulfilled.

It is known that aliphatic diamines can bind strongly to DNA[36], and thus can stabilise that part of the DNA helix not covered by a histone. The discovery that the disulphide forms of the aminothiol protectors also bind strongly to DNA[37] could explain the need for both the amino and free thiol groups of these protectors.

Thus radioprotective aminothiols, if in fact they do function by binding and stabilizing the DNA helix not covered by histones as suggested by Brown, would produce the following effects:-

1) prevent to some extent primary lesions,

2) by holding in place the loose ends from a single strand rupture, lessen the secondary damage arising from physical shortening or chemical alteration, and

3) increase the time for DNA replication so that repair can proceed before replication takes place.

The discovery that histones themselves contain thiol groups[38] has induced Brown to consider a modification of his hypothesis to include Eldjam and Pihls'[22] theory of mixed disulphide formation alluded to above. BAL and DTT instead of an amino group have an extra thiol group. It is possible that they act by forming disulphides with the histones and thus stabilise and protect the molecule from radiation. It has been shown that one of the most important structural requirements of the active aminothiols is that the amino and thiol groups should not be separated by more than 3 carbon atoms[39]. In BAL, the two -SH groups are adjacent and in DTT there are only two c atoms separating them.

The presence of sulfhydryl or potential sulfhydryl groups is not always sufficient to confer protective properties on the substances containing them[1]. One would expect thioctic acid (α - lipoic acid, an important physiological substance) to behave in a similar fashion to BAL or DTT. In the reduced form it has two -SH groups and a low redox potential[47], which would be expected to confer on it radioprotective properties. However, results from different laboratories are conflicting, possibly due to its varying solubility at different pHs. Some workers found that it was about twice as effective as cysteamine in protection against Xrays[40]. If administered to rats and mice before X-irradiation, it was effective in lowering the mortality, preventing loss of weight and raising the leucocyte count, which drops significantly in animals even after small doses of Xrays[41,42]. Other experiments, however, show a very small protective effect[43] or none at all[44].

Bacq and Alexander[17] still incline to the view that the mechanism of protection by sulfhydryl compounds in mammals at the molecular level involves the interaction of SH groups with radicals themselves and that processes such as instantaneous repair of the primary lesion by hydrogen transfer are ultimately responsible for the observed increase in radioresistence.

Another possible way in which sulfhydryl compounds could reduce radiation damage has recently been put forward by Maisin and Lambiet[45]. They found that when radioprotectors such as AET (2 β aminoethylisothiourea), glutathione and serotinin were injected into mice, there was a drop in the number of divisions in the stem cells of the small intestine due

to a prolongation of S, G_2 and M of the cell cycle. They concluded that the injection of large doses of thiol compounds decreases the metabolic activity of cells and initiates a sequence of intracellular changes which result in an increased radioresistance in these cells.

Conclusion

Dithiothreitol produces a significant reduction in radiation-induced damage in marsupial cells in tissue culture. The chromosomal damage (scored as the number of breaks per nucleus) is reduced by half when DTT is present. The fact that it is odourless and is fairly resistant to oxidation by air may make it preferable to the thiols already in use as protective agents. It would be of interest to compare the above results with the effects of BAL operating in the same system under the same conditions. Experiments to determine the speed and duration of the protective action of DTT and the effect of this radioprotector on the organism as a whole would be of value.

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G. Modification by Chloramphenicol of Radiation-Induced Chromosomal Damage in Cultures of Marsupial Leucocytes

Introduction

In a study of the rejoining of breaks produced by Xrays in Vicia faba, Wolff[1] found that the healing process was delayed if chloramphenicol, which inhibits protein synthesis, was present between two doses of irradiation. He obtained the same results with the antibiotic aureomycin, also an inhibitor of protein synthesis, but penicillin, which has no effect on protein synthesis, produced no delay in the repair of breaks. He concluded that the inhibition of protein synthesis can keep breaks open.

More recently, Wolff[2] (1964) has presented further evidence which shows that repair occurs in parts of the cell cycle in which there is no DNA synthesis, and that the bonds formed during the repair of chromosome breaks are of a protein nature.

The antibiotic chloramphenicol (CAP), first obtained from Streptomyces venezuelae[3], has been found to inhibit bacterial growth by blocking protein synthesis at some stage in the transfer of amino-acids from transfer RNA to protein[4]. In mammalian cells, CAP causes disaggregation of polyribosomes and is thought to block the attachment of ribosomes to template RNA or to cause an accelerated or premature release of ribosomes from the template[5].

In contrast to other protein inhibitors, particularly puromycin,

CAP has a rather variable effect on protein synthesis in different species[6]. At concentrations of 10^{-4}M to 10^{-5}M, protein synthesis in bacteria is inhibited up to 90%, but these levels are ineffective for animal tissues[7,8] or yeast[9]. Stronger concentrations have been used with more success to inhibit protein synthesis in higher organisms[10-13]. Taylor[14], using CAP concentrations ten to one hundred times those for bacterial systems, found that protein synthesis in cultures of human cells was greatly reduced.

The wide spectrum of effects produced by CAP in mammalian cells includes inhibition of RNA as well as protein synthesis and a depression of the mitotic rate. A comparison of Taylor's work and that of Kurland and Maaløe[15] with E. coli shows that the sensitivity of RNA synthesis to CAP inhibition is the same for both systems. However, since the inhibition of protein synthesis in human cells is established in a very short time, while the rate of RNA synthesis decreases slowly, Taylor suggested that the latter may be a secondary effect resulting from the coupling of RNA and protein synthesis. Godcham and Herbert[5] found that for mammalian cells the reversible effect of CAP on protein synthesis differed from that in bacteria in three respects:-

(1) higher concentrations were needed for the mammalian cells,

(2) the L(+) threo isomer, inactive in bacteria, inhibited protein synthesis in rabbit reticulocytes although to a lesser extent than D(-) threo form and

(3) in bone marrow cells both protein synthesis and RNA synthesis

were inhibited to approximately the same extent, although the possibility that the inhibition of RNA synthesis was secondary could not be ruled out. Thus there are appreciable differences in sensitivity of inhibition of protein synthesis which are reflected in differences of response by mammalian and bacterial cells to this antibiotic.

The action of CAP seems to depend on its stereochemistry as well as structure: the L(+) threo and DL erythro stereoisomers of CAP have been synthesized but show only about 1/200 the antibacterial activity of the natural D(-) form[3]. CAP has the same carbon skeleton (Figure I) as the aminoacids, phenyl-alanine and tyrosine (Fig. II). These, however, occur in nature in the L(+) form, in which they are utilized by living organisms. It is of interest that a number of antibiotics are known which contain in their makeup a D(-) aminoacid or related structure.

The following experiments were begun to study the effect of CAP on the repair process in broken chromosomes following Xray damage in marsupial cells in culture. Short term cultures of leucocytes of the Tasmanian rat-kangaroo, Potorous tridactylus, were used. This material is eminently suitable for such experiments: the irradiation and CAP treatments can be given without the gross physiological side effects present in vivo; the cells are in a resting state and in complete synchrony at the time of irradiation; and as mentioned previously, the favourable nature of the chromosome complement allows accurate scoring at metaphase after colchicine treatment.

It is generally considered that the number of breaks observed at the time of scoring is only a small fraction of the number of primary

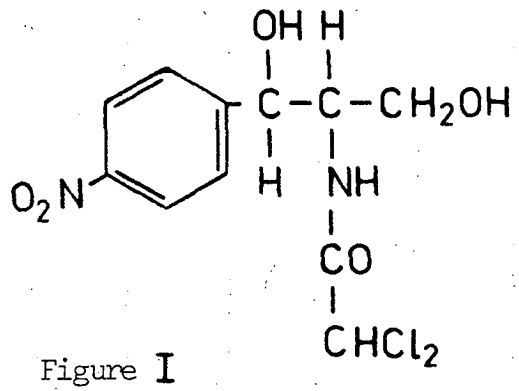

Figure I

D(-) threo chloramphenicol

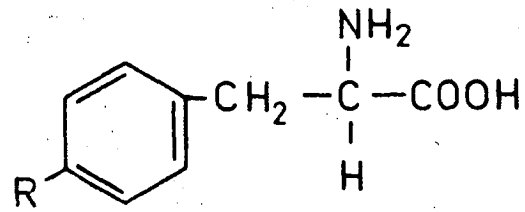

Figure II

L(+) Phenylalanine (R = H)

L(+) Tyrosine (R = OH)

breaks resulting at the time of irradiation[16]. The majority of breaks, with a few exceptions, are thought to be repaired shortly after the dose has been delivered[16]. In order to investigate Wolff's suggestion that CAP is able to influence the repair of chromosome breakage at stages other than DNA synthesis, CAP was introduced for various 24 hourly periods of the culture time (72 hours). No attempt was made to study the effect of the drug during the period immediately following irradiation, where the majority of the repair occurs. Rather, an attempt was made (a) to discriminate periods where the repair of damage was affected by CAP-inhibition of protein synthesis in cells removed from immediate post-irradiation effects and (b) to establish some relationship of the time of repair to the replication phases of chromosomal material.

Since CAP has an inhibitory influence on cell division, presumably because of its ability to inhibit protein synthesis, the mitotic index, i.e. the percentage of cells in division in cultures containing CAP was also compared with that of controls.

Materials and Methods

Blood was obtained from the potoroo by heart puncture under light anaesthesia using a syringe moistened with heparin, and was aerated with gentle stirring for 30 minutes to ensure oxygenation and to eliminate traces of ether.

The whole blood was irradiated with 200 roentgens delivered in one dose at 252/minute, ($\frac{1}{2}$ value layer 0.5mm Cu; 235 KV Peak; added filter

1 mm aluminium; current 15 mA).

An hour after irradiation, a few drops of phytohaemagglutinin (PHA, Wellcome) were added to 10 ml aliquots of blood which were centrifuged at 100 g for 5 minutes. The leucocyte suspension in the supernatant plasma was removed; the leucocytes separated by centrifuging at 150 g for 7 minutes, washed once in complete medium, and then cultured in medium 199 containing 12 to 14% foetal calf serum (both from Australian Commonwealth Serum Laboratories). PHA was added to give a final concentration of 2% to cultures which were maintained at 37°C during the whole culture period.

The CAP (D(-) threo form, Parke-Davis) dissolved in sterile Hank's balanced salt solution (B.S.S.) was added for definite periods to give a final concentration of 1.5×10^{-3}M. Parallel controls received an equal volume of B.S.S. without CAP. Where necessary, CAP was removed by centrifuging the cells, removing the supernatant and then washing the cells twice in complete medium before reincubating them in fresh medium without CAP. Parallel control cultures received the same treatment.

After 72 hours, colchicine was added to all cultures to give a final concentration of 0.004% for a terminal period of 2 hours in order to increase the number of cells arrested at metaphase. After hypotonic treatment for 10 minutes, the cells were fixed in glacial acetic acid-ethanol mixture (1:3). Slides were made, air dried, dipped in methanol and stained with Leishman's stain for 10 minutes.

Results

Effect on Mitotic Index

If CAP was maintained in irradiated and non-irradiated cultures for the whole 72 hours of the culture period, only one or two cells in division could be found per 1,000 cells. When irradiated cultures were treated with CAP for the last 24 hours of the culture period, the mitotic index (M.I.) was approximately 1/3 of that in parallel cultures without CAP (Table I). Some decrease in the M.I. occurred if CAP was present for the first or second 24 hour interval. However, washing procedures used to remove the drug further depressed the rate of cell division in irradiated cultures since this tended to selectively eliminate some of the cells more acutely damaged by Xrays.

Table I

Effect of CAP on the M.I. of potoroo leucocytes after 72 hours in culture, with colchicine treatment for a further 2 hours

Xray Dose at 25r/m	Cells Counted	CAP	M.I.
200r	10,000	none	1.82%
200r	10,000	CAP present for the final 24 hrs of culture	0.62%

Effect on Rejoining of Broken Chromosomes

The chromosomal damage in irradiated cultures with CAP present for different times of the culture period, also the damage in parallel irrad-

iated cultures without CAP, is shown in Table II.

Since the cells were irradiated in G1 before the beginning of the culture period, the damage produced would be expected to be chromosomal[17]. Any chromatid damage present at time of scoring would be a result of spontaneous aberrations arising under culture conditions. This was borne out by the results which show the chromatid damage in all cultures to be fairly constant and of the same order as the chromatid breakage in the unirradiated control.

The damage expressed as the number of breaks/cell was obtained by adding chromatid and iso-chromatid deletions to twice the number of two-hit chromosomal events.

Irradiated cultures which received two washings after 24 and 48 hours respectively (and thus had to be centrifuged three times) showed a lower residual damage than those which were not subjected to any washing procedures. This probably resulted from the elimination or delay of the more damaged cells which are prevented from reaching metaphase in the allotted time and is reflected in the increase in the percentage of normal cells scored for these cultures (Table III).

A summary of the residual breakage after the various treatments is given in Table IV. When CAP was present for the first 24 hours of the culture period, the residual breakage was somewhat greater than in cultures of irradiated cells without CAP. When CAP concentration was maintained for an additional period of 24 hours the residual breakage showed no significant increase over that produced in the first 24 hour

Table II

Residual Chromosome Breaks in Irradiated Cultures with and without CAP. Colchicine added for the final 2 hours of culture period (72 hours)

200r at 25r/m	1 ml CAP - 24 hr periods 1st	2nd	3rd	1 ml BSS - 24 hr periods 1st	2nd	3rd	Cells scored	Chromatid Deletions %	Iso-chromatid Deletions %	Chromosome Two-hit Events	Breaks per cell	Difference
-	-	-	-	-	-	+	100	8	15	0	0.23	0.05
-	-	-	+	-	-	-	100	9	17	1	0.28	
+	-	-	-	+	-	-	100	8	47	6	0.67	0.09
+	+	-	-	-	-	-	100	7	51	9	0.76	
+	-	-	-	+	+	-	100	10	39	5	0.59	0.10
+	+	+	-	-	-	-	100	13	46	5	0.69	
+	-	-	-	+	+	+	100	10	64	4	0.82	0.14
+	+	+	+	-	-	-	25*	12	68	8	0.96	
+	-	-	-	-	-	+	100	10	68	3	0.84	0.19
+	-	-	+	-	-	-	100	9	76	9	1.03	
+	-	-	-	-	-	+	100	10	63	5	0.83	0.18
+	-	-	+	-	-	-	100	10	73	9	1.01	

* M.I. was so low that scoring became impossible

Table III

Effect of washing procedure on percentage of normal cells arrested at metaphase after 72 hours of culture

X-irradiation at 25r/m	BSS containing CAP present	BSS present	Two washings	% Normal cells	No. of cells scored
200r	1st 24 hrs	-	at 24th hour	40	100
200r	1st 48 hrs	-	at 48th hour	47	100
200r	last 24 hrs	-	-	29.5	200
200r	-	1st 24 hrs	at 24th hour	54	100
200r	-	1st 48 hrs	at 48th hour	53	100
200r	-	last 24 hrs	-	42	200
200r	-	-	-	45	100

period. If CAP was present for the full 72 hours of the culture period, the mitotic index was so low that no reliable estimate of the damage could be made. Treatment with CAP during the last 24 hours of the culture period resulted in an appreciable increase in the number of residual breaks. Thirty to thirty-one percent more breaks survive following this treatment than in cultures of irradiated cells not given CAP. The residual damage is significant at $P = 0.0035$. The results for duplicate experiments (shown in table II) show a reproducibility of ±2%.

Discussion

Taylor's[18] earlier work showed that CAP interfered with the spindle formation in newt cells. This toxic effect of CAP on the mitotic spindle also occurs in mammalian cells[14] and does not seem to be related

Table IV

Effect of CAP on chromosomal breakage in Potoroo leucocytes scored after 72 hours' culture period

XRay Dose	TOTAL CULTURE PERIOD			No. of total breaks/ cell	Total breaks per cell in excess of that in cultures without CAP	% of breaks in excess of cultures without CAP
	1st	2nd	3rd			
	1 hr	24 hrs	24 hrs	24 hrs		
200r				0.60		
200r	CAP →				0.09	15%
200r	CAP →				0.10	
200r	CAP →				0.14 Low M.I.	
200r			CAP →		0.19	31%
-			CAP →		0.05	

to its effect on RNA or protein synthesis since it occurs at low levels of the antibiotic. The effect on spindle formation appears rapidly after the application of the drug and results in a transitory increase in the mitotic index. A subsequent gradual decrease in the mitotic index results from the inhibition of protein synthesis induced by the drug. In Taylor's experiments with KB cells treated with 1.6×10^{-3}M CAP, the M.I. dropped about 40% below that of the control. In these experiments with potoroo leucocytes 1.5×10^{-3}M CAP for the final 24 hours of the culture period depressed the cell division rate by almost 66%.

It has been shown by Yamada and Puck[19] that chromosomal condensation

occurs in G2. If the synthesis of structural proteins or enzymes is required for this condensation, then CAP by inhibiting these syntheses would delay cells entering mitosis and consequently lower the M.I.

These experiments were begun at a time when it was thought that leucocytes arrested in metaphase after a 72 hour culture period were still in their first division in vitro[20-23]. Recent work with human leucocyte cultures[24,25] and experiments with potoroo leucocytes in section E of this thesis (which were carried out subsequently to these experiments) have shown that a number of cells reaching metaphase after 72 hours in culture are no longer in their first division in vitro. Divisions in potoroo leucocyte cultures appear as early as 24 hours after their initiation. Thus the length of the potoroo leucocyte cycle must be very similar to that of human leucocytes whose cell cycle can be as short as 22 hours[25], with G2 3 to 6 hours in length, S about 12 hours and 6 to 8 hours for G1[21,25].

Whether the cells arrested at the 72nd hour of culture in these experiments (with a 2 hour colchicine treatment) were in their first division in vitro or not, it is clear that the last 24 hours of their culture period would include G2, the whole of S and some time immediately preceding S.

It has been shown that chromosomes exposed to radiation just before S or in early S, i.e. before much DNA synthesis has occurred, behave as though they had already replicated[26]. A possible explanation is that the protein moiety of the chromosome duplicates before the DNA, and this

duplication results in the chromosome behaving as a double structure to X irradiation. McLeish[27] found in fact that histones of the chromosomes in Vicia faba duplicate in S or just before S. In the potoroo irradiated cultures, the largest residual damage was found when CAP was present at a period which embraces the early stage of S and possibly some hours before its onset. If the repair of chromosomal damage can take place at the time of protein synthesis in the last 24 hours of the culture period, then the increased damage in irradiated cultures in the presence of CAP can be explained.

Without the aid of autoradiography it is difficult to determine at what stage the cells were during the first 24 and 48 hours of the culture period. Seeing that CAP depresses the M.I. by an appreciable amount, it is possible that in cultures treated with this antibiotic, the normal time for cell division is lengthened to such an extent that the majority of the cells arrested at the 72nd hour are still in their first mitosis. Further evidence obtainable by pulse-labelling is necessary to establish this unequivocally; but if we assume that the majority of cells are in first division, the results can be interpreted as follows:-

1. The presence of CAP in non-irradiated cultures does not cause any appreciable chromosomal damage. The small increase in the number of residual breaks (0.05 per cell) can be attributed to an increase in the number of spontaneous breaks held open by CAP treatment.

2. The presence of CAP in the irradiated cultures results in an

increase in the number of residual breaks:

(a) If CAP is present during the early phases of culture period only, the number of breaks remaining open is increased by 15% relative to irradiated cultures without CAP.

(b) If the period of CAP application is extended to cover the middle 24 hours of the culture time, there is no significant increase of damage over that in (a).

(c) If present for the final 24 hours, the number of breaks is increased by about 30% relative to irradiated cultures without CAP.

The repair of chromosomal damage produced by irradiation may involve protein synthesis in several ways: in the repair of actual chromosomal structure; in the synthesis of enzymes required for repair; in re-establishing a peptide linkage; or in the healing of breaks by some unknown substance, which at all events is not DNA or RNA[1]. When labelled CAP is fed to Staphyloccocus aureus, all the radioactivity becomes associated with the ribosomes and soluble fractions[28]. ^{14}C-CAP in cell-free systems is also bound by ribosomes from this bacterial species; the binding is immediate, independent of external supply of energy, unaffected by the incubation temperature, and easily reversible[29]: as soon as CAP is removed, protein synthesis resumes again. Thus it may be possible by using labelled CAP in cultures of leucocytes to investigate the mechanism of repair.

Wolff[16] showed in his experiments that most of the repair of irradiation damage occurs shortly after irradiation. The results of the present experiments indicate that some repair is still in progress

during the first 24 hours following irradiation. A more significant inference is that appreciable repair in the remainder occurs in the last 24 hours of the culture period, i.e. at least 48 hours after the Xray dose has been delivered.

Conclusion

In accord with other experiments on mammalian cells, CAP, at concentrations of the order of 10^{-3}M, has a strong inhibitory influence on the mitotic divisions of potoroo cells in culture. When CAP was present for the final 24 hours of the culture period (i.e. during an interval which would be expected to include all of S and a short period of time preceding it), the M.I. decreased to one-third of that of irradiated cultures without CAP treatment, while the gross irradiation damage was increased by about one-third. However, CAP did not cause any appreciable damage to unirradiated cells.

The above results are interpreted as being due to inhibition of protein synthesis occurring during the final 24 hours of the culture period; a percentage of breaks produced by X-irradiation which normally restitute at this time are prevented from healing by the presence of CAP. These results also indicate that some repair of damage caused by Xrays occurs in this part of the culture period, that is, at least 48 hours after the Xray dose was received.

These experiments give only an indication as to what may be occurring, and should be repeated on cultures in which first divisions can be scored without ambiguity. Autoradiography would enable an accurate

estimation to be made of the type of damage produced and the exact stage of the cell cycle at which repair can be inhibited by the presence of chloramphenicol.

Puromycin would probably be a more suitable protein-inhibitor for these experiments. It is effective at much lower levels in mammalian cells, and as it has no inhibitory action on the mitotic spindle, it would consequently be expected to have less toxic side effects for cells in culture.

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H. Long-Term Cultures of Marsupial Cells

I Setting up Primary Cultures

In 1878, Claude Bernard[1] had pointed out the importance of the internal environment (milieu intérieur) on the regulation and proper functioning of tissues within the living organism. Due to him, this environment came to be considered not only as the product of tissue metabolism, but as something that reacted on the cells themselves regulating their reactions and rate of activity. In tissue culture, the internal environment is replaced by an external medium which provides the necessary nutrients, appropriate pH and optimal temperature range. Recently antibiotics have been included in most media: their presence diminishes the chances of contamination and facilitates aseptic conditions imperative for the successful propagation of cells in vitro.

Aseptic techniques and preparation of the culture medium

The aseptic techniques and their washing and sterilization of glassware etc. are based on the methods outlined in Paul[2] (1959), Parker[3] (1961) and Penso and Balducci[4] (1961). Before any experiment was undertaken the doors and windows of the laboratory were securely closed and ventilators turned off. The benches were swabbed down with 70% ethanol, and a UV lamp turned on for 10 minutes to sterilize the air directly above the bench where media were to be prepared and fresh tissues to be handled.

The glassware was washed with soap and rinsed till clean, then boiled for a few minutes in 7X solution (a detergent available on the American market for tissue culture work). Then followed washing seven times in fast running tap water, two rinses in deionized water, after which the

glassware was dried in an oven at 99°C. Flasks were plugged with cotton wool, the plugs covered with silver paper and the graduated pipettes plugged with 1" of cotton wool placed individually in paper envelopes and sterilized at 160°C for 2 hours. Pasteur pipettes had plugs of cotton wool (these cotton wool plugs remain in situ during the use of the pipettes) and their thin ends were sealed before sterilization. The seal is broken off just before the pipette is flamed for use. Rubber bungs, magnetic stirrers covered with polythene etc. were sterilized, sealed in paper bags by being autoclaved at 15 lbs/sq. inch for 30 minutes. All instruments and syringes were sterilized by boiling in deionized water for 30 minutes just prior to use.

The medium used was Parker's medium 199 from the Australian Commonwealth Serum Laboratories (C.S.L.). This medium contains all the necessary nutrients as well as some penicillin and streptomycin. Horse or foetal calf serum (C.S.L.) was added at a concentration of 8% to 12%. The serum is essential for cells to adhere to the bottom of the culture vessels[5]; if it is omitted the cells remain floating in the medium and no proliferation ensues. At first, horse serum was used, but this tends to develop a fine precipitate after a few weeks which makes it difficult to recognise early stages of bacterial contamination. Foetal calf serum has the advantage of being free of all precipitate for the full three months, the time for which a batch of serum is still usable. Water for making up the 199 medium and balanced salt solution (B.S.S. from C.S.L.) was glass-distilled, sterilized by boiling in a flask with a cotton wool plug for 30 minutes, and then cooled to room temperature. Media, sera etc. kept at 0° to 5°C in

a refrigerator were allowed to warm up to room temperature before use. Aqueous sodium bicarbonate (2.8% w/v, C.S.L.) was used in the proportion of 8 ml to 100 ml of 199 plus serum. This amount adjusted the pH to about 7 determined by the colour of the indicator (phenyl red) which is included in the 199 medium and in the sodium bicarbonate solution. All movement in the laboratory was reduced to a minimum while making up media or carrying out any sterile work, and care was taken that any sterile object that came in contact with nonsterile material was immediately discarded. A small spirit lamp or a low bunsen burner was used for flaming tips of pipettes or necks of flasks and bottles. All culture bottles, centrifuge tubes, etc. when unstoppered were kept on a slant if possible so that the chance of any bacterial or yeast spores dropping in from the air was minimized. Throughout all operations, the fact that the tissue culturalist himself presents the greatest danger of contamination was kept constantly in mind.

A constant temperature room (37°C) and incubators at 37° and 27°C were used for the cultures, and an inverted microscope enabled the surveillance of culture conditions from time to time with a minimum amount of disturbance to the cells. All media were warmed to 37°C before coming in contact with any cells. If the medium in the cultures became too acid, the cork was removed from the flask, the neck flamed and the flask was held at a slant of about 45° to the horizontal for a few minutes to allow the escape of the collected CO_2. Cultures that were too alkaline were gassed with a mixture of 95% air and 5% CO_2 for a few minutes; the medium was then gently agitated until the correct colour of the indicator was

obtained. The neck was flamed again, the cork reinserted and covered with some silver paper to help keep the flasks air tight. It was found that Erlenmeyer flasks, 50 ml, with a completely flat bottom were most suitable for tissue culture.

Dissection of the animal, chelation of tissues and setting up cultures

Whereas nearly all tissues from vertebrate embryos flourish under culture conditions, only a few adult tissues, notably from testis and kidney, can be propagated successfully in vitro. For the purposes of tissue culture, all specimens obtained from animals in the post-natal phase can be considered adult tissues. The differences between old and young tissue seem to be related to the amounts of connective matrix present. Tissues such as kidney, in which there is little fibrous material, behave in practically the same manner irrespective of the age of the donor, whereas fibrous tissues grow much better from young animals[2].

Attempts were made to establish primary cultures from the marsupial rat kangaroo, potoroo (Potorous tridactylus). When the chelating agent was versene (ethylene diamine tetra-acetic acid) in a balanced salt solution free of Mg and Ca ions (made up according to Paul[2], p.182 but without glucose) the cultures proved unsuccessful. However, when trypsin (0.25%, C.S.L.) was used cultures of potoroo kidney and testis cells were obtained at the first attempt.

Ether was first employed to anaesthetise the animal, but later Nembutal (Na pentabarbitone, 60 mg/ml) was found to be more efficient. The animal was weighed and the Nembutal injected intraperitoneally at the rate of 1 ml/4 lbs body weight. The kidneys and testes were removed aseptically

and cut up finely in a petri dish. Small pieces were placed in a flask with a stopper and 5 times the volume of trypsin solution added. This was then stirred mechanically with a magnetic stirrer for 30 minutes. The supernatant containing suspended cells was pipetted off, placed in 10 ml aliquots into stoppered centrifuge tubes and spun for 7 minutes at 100g. The trypsin supernatant was removed by a pipette and replaced by 10 ml of B.S.S. or preferably complete medium. The cells were dispersed by a Pasteur pipette and then centrifuged. The supernatant was removed and the cells washed again as above. (If the washing was done with complete medium instead of B.S.S., then the cells attached themselves to the vessel much more readily on being cultured.) Then complete medium (10 ml) was added to each tube and the cells evenly dispersed with a Pasteur pipette and the concentration of cells was estimated by a haemocytometer.

Culture flasks were filled with 8.8 ml of 199 containing 12% serum and inoculated with the cell suspension to give a resulting cell count of 300,000 to 500,000 cells/ml. The flasks were stoppered with sterile rubber bungs, covered with silver paper to prevent any escape of CO_2 and placed in an incubator at 37°C. After 24 hours, a number of cells could be seen attached to the bottom, some already forming germinating centres where a few cells had already divided. After 48 hours the medium was removed with the debris and unattached cells and replaced by 199 containing only 8% serum. Both the testis and kidney cultures gave prolific growths of healthy-looking fibroblasts with very clear cytoplasm and the cells completely covered the bottom of the vessel in 4 to 5 days.

Subculturing

To subculture, the medium was removed and replaced by 8 ml of versene solution and the flasks were warmed on a waterbath (37°C) until the cells became detached. The cell suspension was centrifuged (100g for 6 minutes), the versene removed and replaced by 1 ml of complete medium and the cells evenly dispersed by pipetting up and down. One third of this cell suspension was inoculated into a fresh flask containing 8.8 ml of complete medium (i.e. 199 with 8% foetal calf serum). The new cultures were ready for subculturing in 3 to 4 days. It was found, however, that the removal of the cells with versene was difficult and often up to 55 minutes of versene incubation was necessary before most of the cells became detached. Even using a policeman (a piece of glass rod covered with rubber tubing at one end) to scrape the bottom was not successful. When trypsin was substituted for versene the fibroblast cells came off in 2 to 3 minutes. The trypsin was removed by centrifuging and the cells were washed in complete medium to remove the enzyme before being inoculated into fresh vessels. In a couple of subcultures, the cells had lost the vacuoles in the cytoplasm which had appeared when versene was used for long periods and the cytoplasm became completely clear. During this time, in spite of the initial difficulty of removing the cells with versene, the rate of proliferation did not subside and the cells grew at an unabated rate.

About 7 weeks after the initiation of the primary cultures, the kidney cells became more and more difficult to remove with trypsin. Many of the cells became rounded and more epithelial in appearance and even 60 minutes of trypsinization sometimes failed to move the cells. This pro-

longed trypsin treatment proved injurious to the cells and when subcultured the damaged cells produced a great deal of debris which had to be removed before addition of fresh medium 24 hours after subculture. When versene was tried the kidney cells became detached in 5 to 10 minutes, and after a few subcultures again regained their healthy appearance; but by now all the cells were epithelial, a morphology they have retained after almost 4 years of culturing.

It is difficult to determine whether the kidney cell population originally consisted of over 90% fibroblasts with only a few epithelial cells which under culture conditions gradually prevailed, or whether the kidney fibroblast-like cells changed after 7 weeks to an epithelial type. It seems clear, however, that the type of attachment of the epithelial and fibroblast cells is different and different chelating agents act preferentially on the two cell types.

The testis cells presented few difficulties in subculturing and remained fibroblast and sensitive to trypsin removal as previously. When the old fibroblast cells die, they become easily detached and float off with the old medium, while new fibroblasts take their place. As a result of this the testis fibroblast cells could be kept at 37°C for a number of weeks without subculturing. The epithelial kidney cells, on the other hand, are more firmly attached to each other than to the glass vessel, and when a percentage of them die and become detached from the surface of the flask, the whole cell sheet is affected and rolls up to float off into the medium. Once detached, the cells are non-viable and disintegrate and death follows within 24 hours. The only way to save such a culture is to

break up the rolled up sheet of cells into single cells and small cell aggregates by strong pipetting. This apparently enables the healthy cells to free themselves and resettle on the bottom, become attached and start proliferating again.

Different concentrations of serum were tried and it was found that 20% serum was inimical to cell growth. Some cells became rounded, vacuoles appeared and the cytoplasm was studded with fine granules. Below 5% serum, cell growth is retarded and divisions decrease. 8 to 10% serum proved optimal and 8% was adopted as a standard concentration in subsequent work. HeLa cells, on the other hand, which were handled initially in the laboratory, when tissue culture techniques were studied, grew quite well at the higher serum concentrations.

If after subculturing, the flasks were kept at room temperature (25°C), hardly any cells attached. When the cultures were replaced in the incubator at 37°C, however, the cells attached to the vessel and after a short initial lag the cells proliferated again at the previous rate.

II Initiation and Characteristics of Long-term Cultures

The long-term cell line is by definition capable of continuous rapid multiplication in a system maintained *in vitro*[6], a characteristic it has in common with malignant tissues since these can be perpetuated indefinitely under culture conditions. It is usual to allow 6 months to a year to elapse after the initiation of the primay culture before it is considered an established cell line. The cells that proliferate continuously *in vitro* grow in monolayer form. From the moment of being cultured, theybecome

dedifferentiated giving two main morphological cell types, fibroblasts and epithelial cells[7]. It is a striking fact that the cultured cells usually indicate no evidence of behaving in the histotypic manner of their immediate ancestors in the tissue of origin. Some workers have reported the reformation of three dimensional tissue architecture[4] and the retention of certain differentiated characteristics that the cells had originally possessed in vivo[5,6,7]; but in most cases, the differentiated character manifested was not hereditary and disappeared in the subsequent generations.

Champy[8] noted that tissues normally possessing no appreciable mitotic activity in vivo under tissue culture conditions began to proliferate and show an appreciable mitotic index. He attributed this to the sudden absence of inhibitory influences normally applied through intercellular cooperation within the tissue matrix. In support of this proposition he was able to point to his own observation that glial cells of the retinal cultures entered the stage of rapid propagation only on the death of the neurons to which they were attached[9]. In addition he considered that stimulation of vegetative growth by the nutrient and perhaps physical features of the tissue culture environment were important factors.

The life-span of the short-term primary culture is terminated by death or transformation to a long-term cell type. There exists a certain point in time which is clearly the demarcation between the two types of culture, the point at which the slower-growing short-term cells are suddenly overgrown by a morphologically distinguishable, rapidly proliferating new cell type which possesses indefinite propagative capacity. This

phenomenon has been called transformation[6]. A short-term culture once established will proliferate steadily for some time, usually for several months. Then the mitotic index drops and the cells may be in their respective vessels, apparently quiescent, for days or even weeks, until a wave of necrosis finally spreads across the monolayer and the cell line is lost. The alternative to such extinction is transformation[6]: one or several of the cultures may display a nest or two of healthy cells, the beginnings of a new cell type which then recolonise the vessel surface. Most striking of all is the generality of the observation that the altered cell type appears almost simultaneously in several parts of the vessel[6]. The question of what transforms this quiescent pre-existent cell, ancestor of the rapidly proliferating long-term cell type, is still not answered.

Human diploid fibroblast strains, which never transform spontaneously form an exception to the generality of mammalian lines. After a period of active multiplication generally less than a year, human diploid cultures begin to exhibit a lengthening generation time and finally total degeneration sets in[10]. Although diploidy can persist for a few months or even a year in primary cultures of non-human origin, when transformation supervenes the subsequent long-term cultures are almost all heteroploid in karyotype. The onset of chromosomal abnormality is an event which may begin simultaneously in a considerable number of cells soon after the initiation of primary cultures, i.e. during the first few divisions in the new environment. On the other hand, aneuploidy and heteroploidy may set in only about the 40th passage[11] or just prior to transformation. The diploid human strains do not exhibit alterations in karyotype and

show characteristically only about 2 to 3% tetraploidy[12]. It is perhaps this inability to change their original karyotype that inhibits the emergence of a cell type adapted sufficiently to the existing conditions to allow indefinite proliferation. The human diploid cell lines thus retain their mortality as they would had they remained part of the living organism. Actually, no cells having the karyotype of the tissue of origin have been observed to multiply in vitro longer than the lifespan of the animal species from which the tissue was obtained[10]. However, human diploid strains can be readily transformed into long-term cell lines by the introduction of viral infection such as SV40[11].

Malignant cells invariably possess karyotypes which have changed significantly from the normal chromosomal complement of the species in question. Strains of HeLa cells (human carcinoma cells) have stemlines closer to the tetraploid number ranging from 68 to 90 and recently numbers as high as 138 and 148 have been obtained[13]. Besides the property of perpetual propagation, both long-term cultures and malignant cells thus exhibit heteroploidy and aneuploidy in their chromosomal complements. The phenomenon of alteration of a primary cell strain to a permanent cell line is regarded as important by Hayflick because in its simplest terms, it can be regarded as oncogenesis in vitro[10]. Many transformed cell lines will produce tumours if injected in sufficient numbers into healthy animals, but not all long-term cultures possess this property[6].

Observations on growing mammalian diploid fibroblasts have revealed some restriction on the freedom of movement of proliferating cells, which is wholly or partly responsible for the tendency of diploid strains to

form single layers of cells. This directional prohibition of movement is known as contact inhibition[14]. After junction of two opposing sheets of cells, divisions decrease until all growth ceases. All primary cultures exhibit this contact inhibition, a property which has been lost by malignant cells and most long-established cell lines, which proliferate to form several layers at the bottom of the culture vessel. There is, however, the instance of a well established mammalian cell line, 3T3, which has remained extremely sensitive to contact inhibition although it has been propagated in the laboratory for a number of years[15].

Experimental

26-VII-63 Primary cultures of potoroo kidney and testis cells were set up in the laboratory and quickly grew as fibroblast-like cells with clear cytoplasm. They were subcultured (the contents of one vessel into three fresh ones) every 4 to 5 days. Great difficulty was encountered in removing cells with versene and even versene treatment of 50 minutes failed to dislodge all the cells.

20-VIII-63 When trypsin was tried instead of versene it was found that both the kidney and testis fibroblasts were removed in as little as 3 minutes.

8-X-63 Kidney cells began to develop an unhealthy appearance with granular and vacuolated cytoplasm, and their rate of proliferation declined. The testis cells appeared healthy enough, but the colonies that formed after subculture were fairly static and hardly increased in size.

14-X-63 It was thought that the trypsin treatment was proving too drastic and causing the unhealthy appearance of the cells and the decline in the rate of multiplication. Versene was used for a while instead, but was ultimately abandoned since some of the testis fibroblasts failed to become detached even after 1½ hours treatment with versene.

24-X-63 Patches of healthy-looking cells appeared in the kidney cultures and began to multiply rapidly. Most of the healthy kidney cells were epithelial in type and grew in smooth concentric patches. This was probably the point in time when transformation occurred in the kidney cultures. The transformed epithelial kidney cells appeared in 4 culture bottles at almost identical time - there were several patches of the transformed cells in each bottle.

The testis cultures at this point were still fairly static.

5-XI-63 Kidney cultures were now completely epithelial and designated HPK1. Versene was used from now on to remove these cells and it did so in 5 to 10 minutes with incubation in a water bath at 37°C. (Trypsin failed to dislodge all the kidney cells even after 1 hour of treatment.)

The testis cells began to multiply rapidly again, but remained fibroblast-like in appearance. It was assumed that the testis cells had transformed also, and the line was called HPT1. The testis fibroblasts could be removed by trypsin within half a minute; versene was deemed unsuitable and even 1½ hours of incubation with versene only dislodged a portion of the fibroblast cells.

Both testis and kidney cultures were subcultured by dividing the cells from one culture into 3 new vessels of the same size. The new

flasks were ready for subculturing within 2 to 4 days.

To determine the chromosome numbers of the lines, colchicine (.04%) was added ($\frac{1}{2}$ ml to 10 ml of medium) to the culture for several hours. The cells were then detached with either versene or trypsin. After centrifugation of the cell suspension (100g for 6 minutes), the chelating agent was removed and replaced by 1 ml of 199 medium or 1 ml of B.S.S., then 2 ml of warm (37°C) distilled water was added. The cells after being gently dispersed in the hypotonic solution with a Pasteur pipette were then placed in a water bath (37°C) for 10 to 12 minutes. An equal amount of fixative (1 part of glacial acetic acid and 3 parts of absolute ethanol) was added to the cell suspension, and after 30 minutes this suspension was centrifuged, the supernatant liquid removed and 1 ml of fresh fixative was added. After a further 30 minutes, slides were made by placing a drop of cell suspension and a couple of drops of 60% acetic acid on a clean slide and warming the slide over a bunsen flame or on a slide oven at 60°C. The slides were then stained with Leishman's stain.

The epithelial kidney cells have formed a stable line and over a three year period the cell-type pattern has remained perfectly static. Virtually no aneuploidy has appeared: in one hundred metaphases only 1 or 2% of cells show 12 instead of 13 chromosomes, but as it is always one of the smallest chromosomes which is missing, this is probably due to washing out of the smaller elements during hypotonic treatment. This cell line, HPK1, presents an unusual feature inasmuch as after almost four years in the laboratory there are still about 90% of diploid cells (Table I). (Mr. K. Brown who has had the HPK1 line for over 6 months at Lucas

Heights and has maintained the cells at 37°C, reports that the diploid content has been between 92 and 95% after 2 hours of colchicine treatment.)

Table I

Cell Line	%2n	%4n	%8n	Cells counted	Hours of colcemid treatment	Weeks after initiation of cultures	No. of passages
HPK1	87%	11	2	100	25	54	100
HPK1	89	10	1	200	17	114	210
HPK1	90	10	0	100	2	188	300
HPT1	42	56	2	200	17	14	20

The potoroo testis fibroblast line (HPT1) was much less stable and after 14 weeks (20 passages) the number of diploid cells had dropped to about 40% (Table I). The spontaneous aberration of the two potoroo lines seemed to be almost identical, the observations being made, however, at different ages of the two cultures (Table II).

Table II

	Acentric pairs	Chromatid deletions	No. of passages
HPT1	13	2	20
HPK1	12	2	300
HPK1	12	7	320

Aneuploidy in the HPT1 line was also much higher: about 8½% were aneuploid and a percentage of these had extra chromosomes present in the complement;

2% of the cells had one over the tetraploid number, i.e. 27 chromosomes, and on analysis of the complement it was found that 3 instead of 2 Xs were present.

The HPK1 cells have kept not only their diploid chromosome number, but also their contact inhibition - the cells form a perfect monolayer and there is no overlapping or tendency to crowd as with malignant cells or long term cultures. There is a recorded case of an established mammalian line (3T3, which was virus-transformed) that has kept its contact inhibition, but this line of cells is aneuploid[16]. The cell line PTK1, which was established in California by Whalen in October 1962 from kidney of female potoroo[17], had samples of its cells frozen at a tissue culture passage 66. The cells were recovered on Sep. 14, 1965 and it was found that 56% were now hypodiploid with 11 chromosomes instead of 12[18], being monosomic for the small chromosome 5. Thus the stemline number was now 11 with a considerable number of cells in the region of the double stemline number of 20[18]. From a study of a number of idiograms, the arm ratios were also found to have altered considerably[18]; this potoroo epithelial kidney line would thus appear to be far less stable than ours. Although no actual measurements have been done on the chromosome lengths of our HPK1, no obvious changes in relative arm lengths seem to have taken place by translocation of chromatin material, and the chromosomes after nearly 4 years and more than 300 passages in the laboratory appear identical with those at the time of the initiation of primary cultures. This constancy of karyotype should make HPK1 a useful cell line to exploit in the laboratory. It is of interest that permanent cell lines of the marsupial

mouse, Antechinus swainsonii that have been recently established by Moore and Wren[19], all exhibit aneuploidy: there are two near-diploid lines, two near-triploid, one near-tetraploid and one with a variable karyotype. Thus, it seems that marsupial permanent lines like those of placental mammals do not retain their diploid character, and our HPK1 line is exceptional in this respect.

As there were no facilities for autoradiographic studies in our laboratory, Mr. Keith Brown at the Australian Atomic Energy Commission at Lucas Heights very kindly performed an autoradiographic study of the cell life cycle of the HPK1 line and obtained (Fig. 1) a generation time of 22.5 hours. This is in accord with our observations: under optimal conditions an inoculum consisting of 1/4 of a culture bottle will cover completely a fresh vessel in 3 days which means that the cells double in number in $1\frac{1}{2}$ days. As this time includes the initial lag period and the slowing down of growth when the cell patches become confluent, it would appear that the doubling time of the cells during their logarithmic growth phase would be around 24 hours.

III Temperature Effects

The HPK1 and HPT1 lines were kept at 37°C at which temperature the cells grew well and exhibited clear cytoplasm. Fully grown cultures could survive at room temperature (22° to 25°C) for one month without change of medium. At this lower temperature, the rate of cell death exceeded that of cell division and when cells died and floated off, spaces remained which were not recovered by new growth. When such cultures were replaced

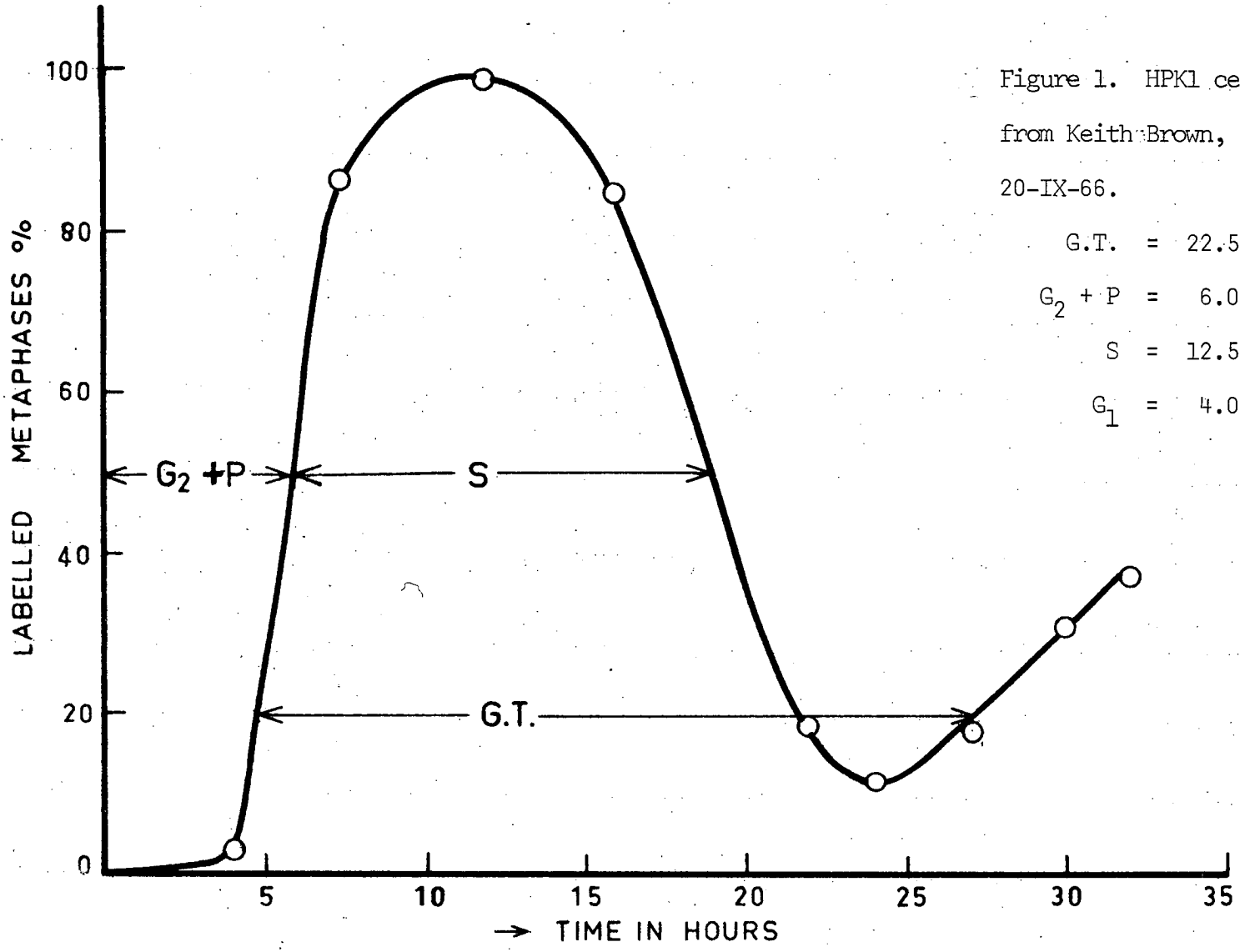


Figure 1. HPK1 cell life cycle from Keith Brown, Lucas Heights, 20-IX-66.

G.T. = 22.5 hrs

G_2 + P = 6.0 hrs

S = 12.5 hrs

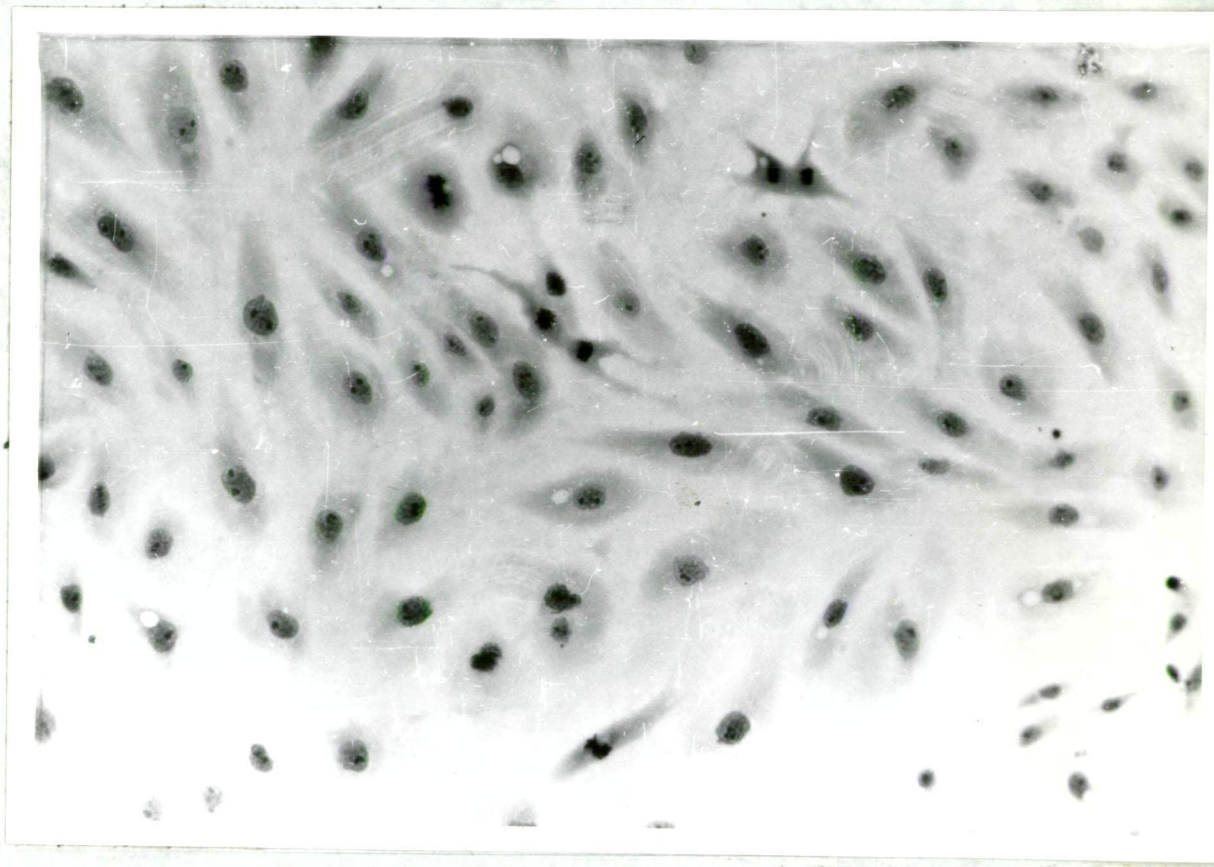
G_1 = 4.0 hrs

in an incubator at 37°C, the rate of cell multiplication returned to normal in a couple of days and the cells were ready to be subcultured.

At 27°C, the cultures could be kept indefinitely with a change of medium only once every month. Cultures have been kept as long as 20 months without subculturing, but after 14 months at 27°C spaces appear between the cells since the rate of cell proliferation does not keep pace with that of cell death. When such cultures are maintained for 4 weeks at 30 to 31°C, the bottom of the vessel again becomes covered with thick cell growth and the cells can be maintained at 27°C for another year or more. This has proved an extremely easy way of keeping a certain number of the cells in reserve in a fairly static condition without much time being spent on their feeding and subculture.

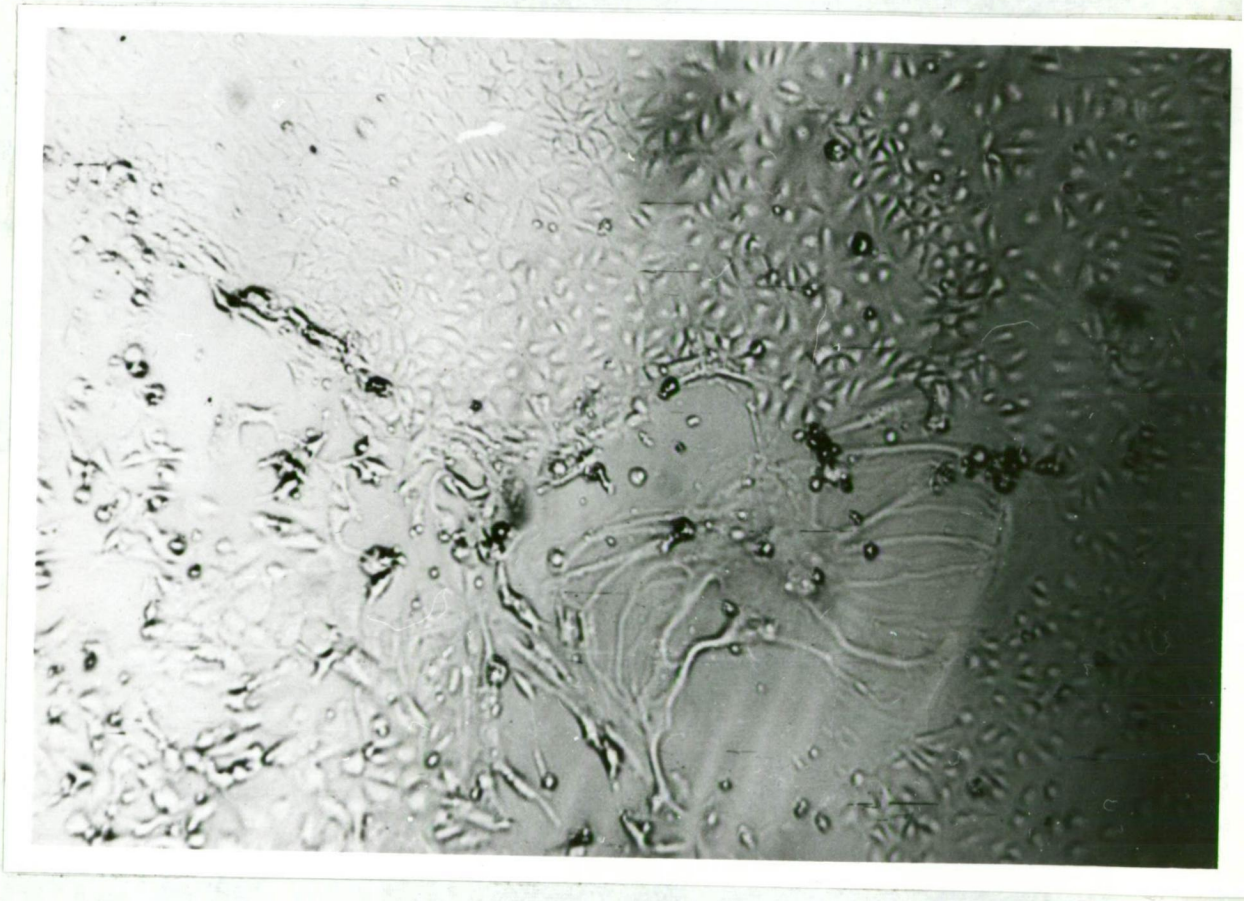
The morphology of the epithelial HPK1 line did not alter appreciably during prolonged periods of incubation at lower temperatures. A few slightly larger cells (probably tetraploid in chromosome number) became noticeable after some months, but these disappeared from the population once the temperature was raised to 37°C and the cells were subcultured two or three times. HPT1 cultures on the other hand after a month at room temperature gradually produced about 10% of cells with a number of long processes resembling dendrites of nerve cells. The cell size, however, remained the same at 22 to 25°C. After 5 months at 27°C, the testis fibroblasts (HPT1) developed a small percentage (1 to 2%) of giant cells with large nuclei and numerous outgrowths of the cytoplasm which gave them a stellar appearance (Photos 1 & 2). The size of the normal fibroblasts was about 88μ x 22μ (an average obtained by measuring with a

Photo 1



HPT1 cells grown in Falcon plastic petri dish. Even without any colcemid treatment, the culture shows a good mitotic division rate

Photo 2



Giant cell from a culture of HPTl cells kept at 27°C for 6 months. With the protoplasmic processes, it measures about 1,000μ across.

graduated ocular device the length and breadth of 100 cells). Smaller cells packed closer together in germinating centres had a more epithelial shape, being 66 to 56μ x 22μ and some 60 x 28μ. Some of these enlarged cells appeared to be about twenty times the size of the ordinary fibroblast. It would seem that at low temperatures, potoroo fibroblast cells undergo endoreduplication a number of times to produce these giant cells. When the temperature was raised to 37°C, the large cells within a couple of days disintegrated and disappeared; apparently their slowed-down metabolic processes were no longer able to cope with an increased rate of turn-over imposed by raising the temperature.

Experiments were begun on synchronizing the cells by using cold shock treatment. The HPT1 line appeared to be suitable for this experiment. It had a high mitotic rate and in the logarithmic growth phase, mitotic indices as high as 3% were obtained without any colcemid treatment. The other advantage presented by the testis fibroblasts was that the cells in mitosis possessed a different refractive index and appeared when viewed under the inverted microscope more rounded and golden and in most cases the chromosomes could be seen arranged on the metaphase plate. This meant that the number of cells in division could be scored without sacrificing the cultures and without the trouble of fixation and slide making and staining. Circles were drawn on the bottom of the glass vessels enclosing about 400 cells. Areas including at least 2,000 cells were scored for cells in division for each experiment. The cultures were given 1, 2, 3, 5 and 15 hours cold treatment at 3.5°C and then returned to 37°C. The mitoses were scored before and straight after the cold

treatment, and then about every hour after the cultures were returned to 37°C until the mitotic index dropped to normal (Fig. 2).

Low temperatures had been successfully used to synchronise about 80 to 90% of HeLa populations by subjecting them to 1 hour at 4°C then restoring the temperature to 37°C[20]. Such cultures showed a gradual decline in the number of mitoses during the first 10 hours after chilling and the mitotic figure reached a minimum, then there was a rapid rise in the number of mitoses and at 17 hours after treatment up to 95% of the cells divided within 1 hour. In every experiment this rapid period of cell division had been preceded by a rise in the mitotic index of the culture. The greatest mitotic index recorded varied between 3.5 to 8%, but never exceeded this latter value[20].

Besides the obvious value in determining the percentage of synchronisation of potoroo fibroblast cells and how this is affected by the initial mitotic index of the cultures, it was considered of interest to see in what way the time (after cold treatment) at which the highest cell proliferation occurs is dependent on the length of the normal cell cycle. No cell counts were performed at this stage of the project, but it can be assumed that the highest peak in the mitotic index after cooling just precedes the sudden rise in cell numbers as was the case in the HeLa experiments outlined above. From Table III, it can be seen that the minimum in cell division occurs somewhere between 1 and 2½ hours and the maximum between 3½ and 6 hours after chilling. Thus it would seem that the potoroo testis line recovers more quickly than the HeLa cells from exposure to the same amount of cold treatment. It is possible that the

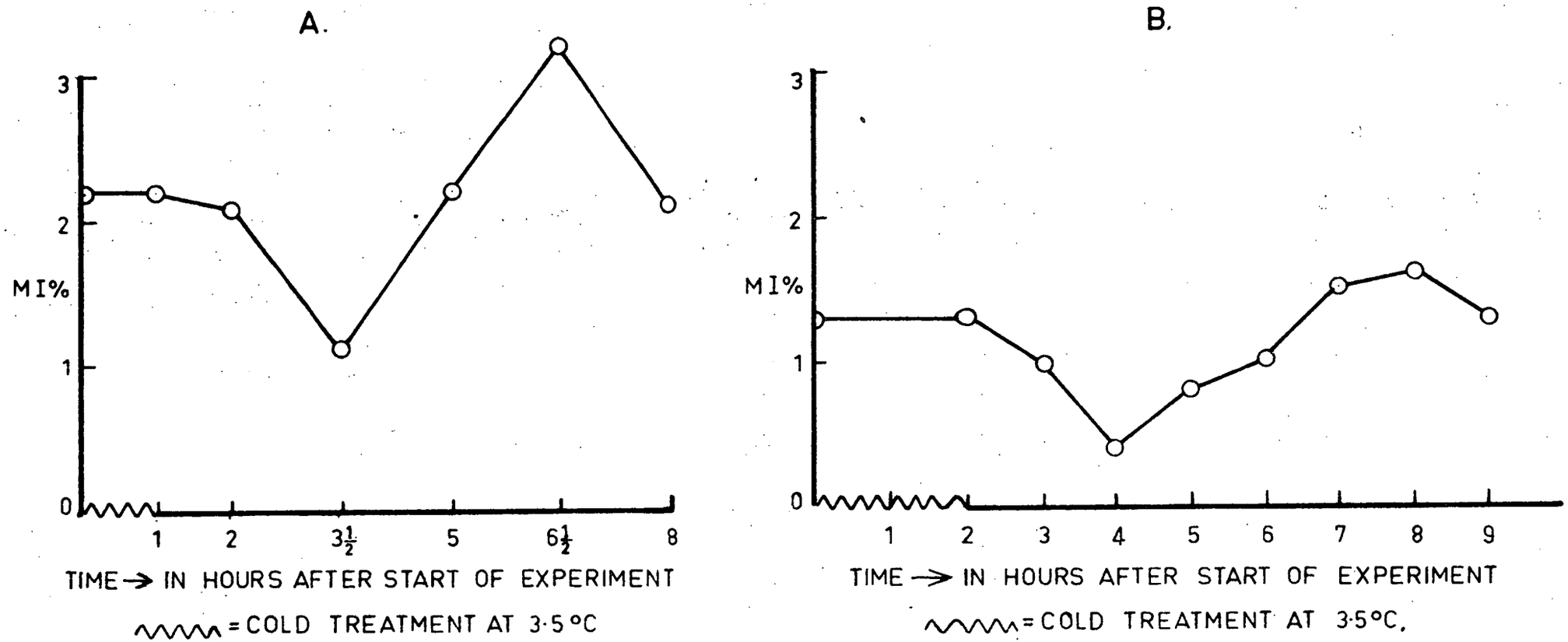


Fig. 2. Mitotic indices of marsupial cells (HPT1) subjected to cold treatment

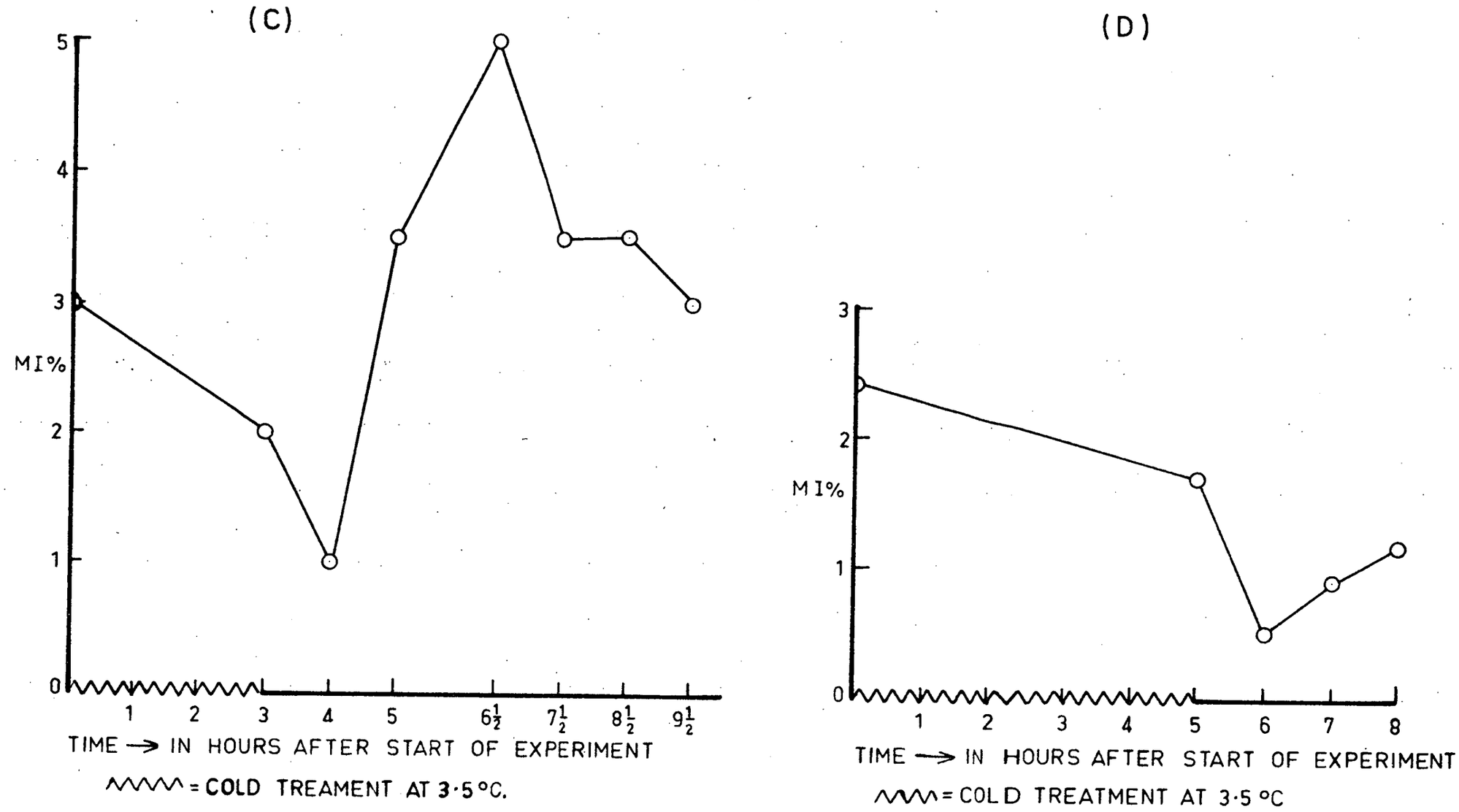


Fig. 2. Mitotic indices of marsupial cells (HPT1) subjected to cold treatment

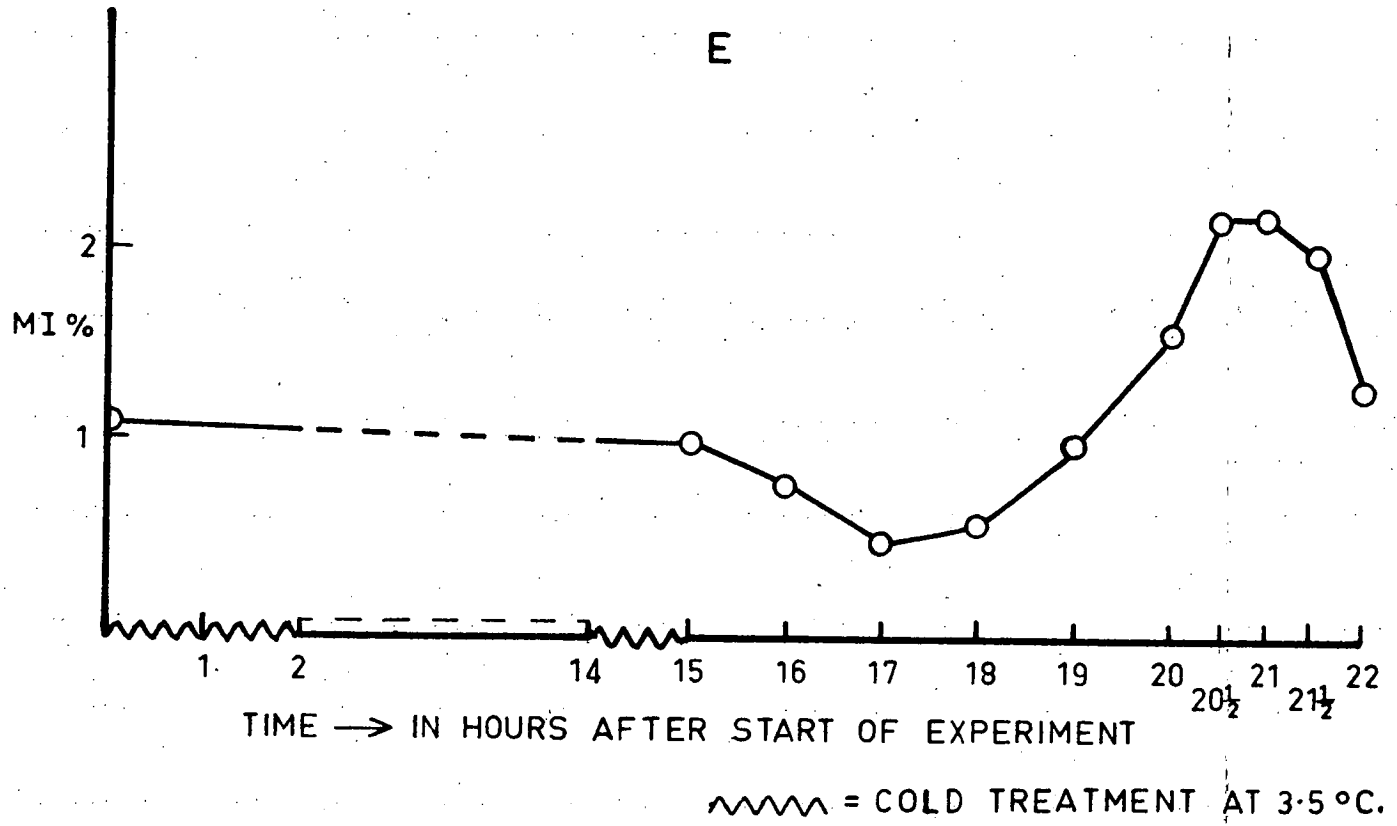


Fig.2. Mitotic indices of marsupial cells (HPT1) subjected to cold treatment

minimum and maximum mitotic peaks for potoroo testis cells occur earlier than in the HeLa populations because the potoroo fibroblasts may have an appreciably shorter life cycle.

Table III

Initial M.I. HPT1 cultures	Time of cold treatment	Minimum M.I.	Time of min. after cold treatment	Maximum M.I.	Time of max. after cold treatment
2.2%	1 hour	1.1% down by 50%	2½ hrs.	3.3% up by 55%	5½ hrs.
1.3%	2 hrs.	0.4% " " 69%	2 hrs.	1.6% " " 23%	6 hrs.
3.0%	3 hrs.	1.0% " " 66%	1 hour	5.0% " " 66%	3½ hrs.
2.4%	5 hrs.	0.5% " " 79%	1 hour	- -	-
1.1%	15 hrs.	0.5% " " 55%	2 hrs.	2.2% " " 100%	6 hrs.

After 15 hours at 3.5°C (Fig. 2, E), the metaphases looked shrunken and some could be seen floating off and disintegrating in the first hour at 37°C. However, even after this prolonged time at low temperatures, the cells recovered in 6 hours to give a maximum in the mitotic peak. If we disregard culture B (figure 2) which was older than the others and probably no longer in logarithmic phase growth, as most of the spaces had been filled with cells, then it would seem that the length of time of cold treatment is a function of the amount by which the peak of the mitotic index surpasses the initial rate of division.

After two or three subcultures, all the descendents of the cells used in these experiments, even from the cultures that had received only 1 hour cold treatment, slowed down in the rate of multiplication, so that the

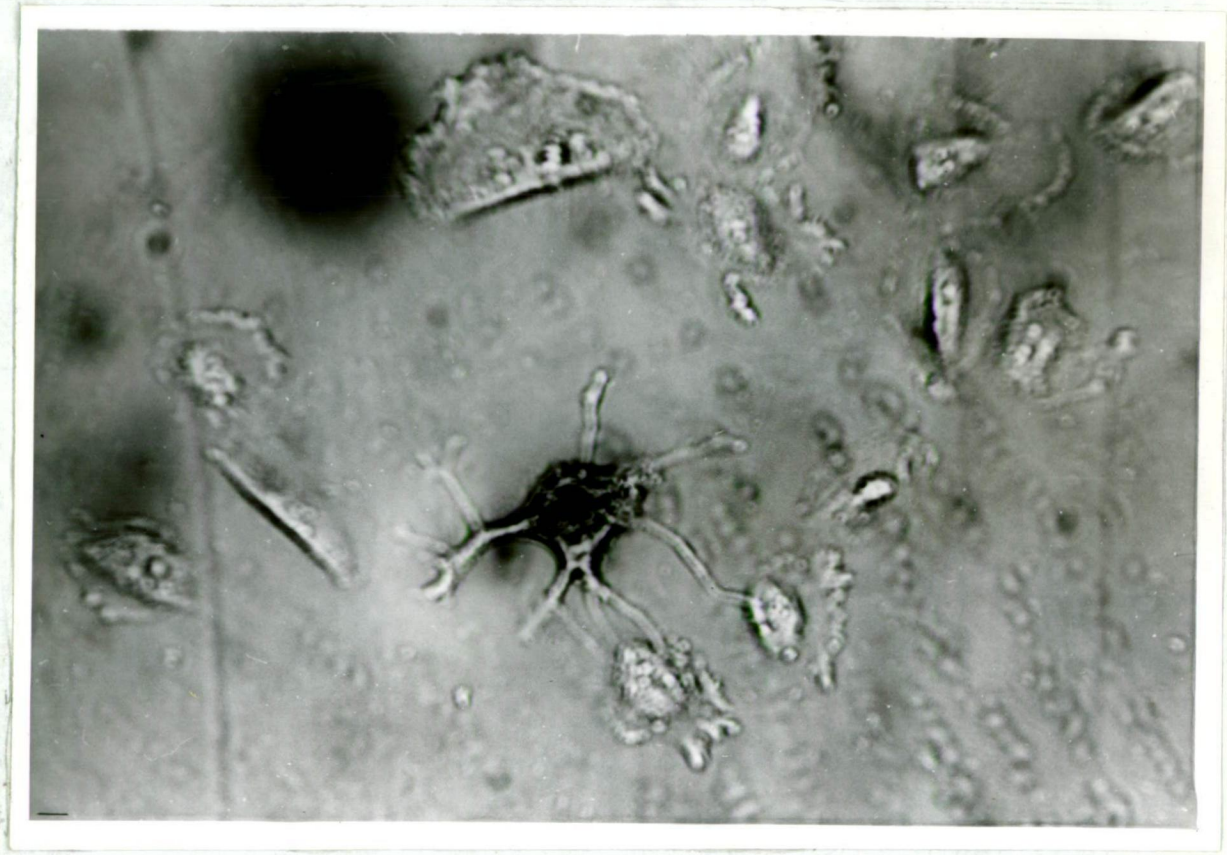
time between subcultures had to be lengthened to about two weeks. The cell morphology gradually altered and in 6 weeks many of the cells had become rounded and nearly four times the size of the original cells. Attempts to ascertain chromosome numbers in these altered cells were unsuccessful. After a few hours of colchicine treatment when the fixative was added, the large cells immediately disintegrated leaving behind only the outlines of their cell wall structure. In ten to twelve weeks there were no original fibroblast-type cells present. Some of the cells had long narrow branching processes (Photo 3), others had grown by endoreduplication into flat, squamous cells about 10 times the size of the original fibroblasts (Photo 4). Four months after the cold treatment, the last of the giant cells disintegrated. Thus, although the potoroo fibroblasts appeared initially to be more resistant to cold treatment than the HeLa cells, their metabolism was so impaired that the whole line became extinct and it was not possible to complete the experiments on their synchronisation.

Breakage in human chromosomes has been observed after cold treatment[21]: the cold breaks showed a non-random distribution over chromosome length. Polyploidy also developed, and it is probable that low temperatures favour cells with high chromosome numbers[21]. On the other hand, mammalian cell lines have been subjected to even longer periods of cold. Mouse L fibroblasts have been exposed to 4°C for periods of several weeks, with only 1 day at 37°C between each treatment. After one year the cells were still viable, but the polyploidy had risen to 46% compared to only 12% in the controls[22].

Photo 3

HPT1 cells showing long cytoplasmic processes - the descendents of cultures that had received cold treatment at 3.5°C

Photo 4



HPT1 cells, descendents of cold treated cultures - cell in centre with dendrite-like process surrounded by large squamous cells

IV Hybridization Experiments

As long as the study of genetic recombination depended entirely on the analysis of the products of sexual reproduction, there seemed few prospects of successful study of genetics of somatic cells. The developments in microbial genetics gave new hope in this field. Hybridization opened up further possibilities: genetic analysis of somatic cells could now theoretically replace experimental breeding and combined with tissue culture, could tremendously accelerate the genetic study of species where experimental breeding is slow, or, as in man, impossible for other reasons[23].

Barski, Sorieul, Cornefert and Ephrussi[24,25,26] succeeded in obtaining the first hybrid line whose chromosomal composition was a summation of the parental karyotypes. The first experiments on hybridization of cells used two lines of mouse fibroblasts (derived from Sanford's so-called "high" and "low" cancer lines) differing in karyotype sufficiently to allow recognition of a certain number of chromosomes from each line. Mixed cultures were set up with even numbers, 2×10^5 cells of each type. The mixed cultures were kept at 37°C and subcultured once a week. Hybrid cells appeared $2\frac{1}{2}$ months after the beginning of the experiment and their proportion rapidly increased in 3 of the 4 mixed cultures. The hybrids had a distinct selective advantage. Clones were established and these were easily recognisable because of the larger size of the cells[27].

Somatic hybrids from interspecific (mouse x rat) matings of cells in tissue culture were achieved[28], and recently by use of UV-inactivated viruses artificial karyotypes of animal cells from different vertebrates

have been established[29]. To form these heterokaryons, differentiated and undifferentiated cells such as rabbit macrophage, rat lymphocytes, hen erythrocytes and HeLa cells were employed. It was found that the cytoplasm plays an important part in influencing the activity of the nucleus. In all cases where a cell which synthesized a particular nucleic acid was fused with one which did not, the active cell initiated the synthesis of this nucleic acid in the inactive nucleus. The nuclei of heterokaryons in which DNA synthesis took place underwent mitosis, and those nuclei which entered mitosis synchronously usually fused together[29]. This resulted in the progressive formation of mononucleate hybrid cells containing in their single nucleus chromosomal complements of different species.

Another important fact to emerge from these experiments is that cells from different vertebrate species are compatible with each other, and the cytoplasmic signals responsible for the control of nucleic acid synthesis cannot be species specific.

According to the work of Roizman[30], heterokaryocytosis which occurs frequently in cultures is often the result of viral infection and is brought about by successive cell fusions between virus-infected and non-infected cells; the viral infection alters the cell surface, contact inhibition is lost and the way is left open for the incorporation of foreign nuclei into the cytoplasm.

As we had in our laboratory HeLa cells (a malignant cell line of human carcinoma which has lost all contact inhibition) and potoroo epithelial cells (HPK1) which although grown in the laboratory for some

years had retained their diploidy and contact inhibition, it was decided to try to hybridize the two lines. Should a hybridization between the two lines take place, there would be no difficulty in recognising hybrids, since the chromosomes of the potoroo are so distinctive that most would act as markers - in particular, the potoroo X chromosome with its heterochromatic region could be recognised at a glance.

Equal numbers of HPK1 and HeLa cells were inoculated into fresh vessels to give a final concentration of about 100,000 cells/ml. The medium was 199 with 8% horse serum and the cells were kept at 37°C. Next day both potoroo and HeLa cells were attached to the bottom, but the HeLa cells were already beginning to overlay the potoroo cells. On the second day, only a few potoroo cells were visible and 90% of the space was covered by HeLa cells. The HPK1 cells, like HeLa cells, are epithelial but their cytoplasm is much clearer and since they form only one layer there was no trouble in distinguishing the two types. After 3 subcultures (3 to 4 days between each), no potoroo cells could be found and when the cells were given hypotonic treatment, fixed, slides made and stained, only HeLa cells were found to be present with their aneuploid human chromosome numbers.

In the next experiment, potoroo cells were grown first until half the area of the culture vessel was covered by them, and then an appropriate inoculum of HeLa cells was added; but after the second subculture, no potoroo cells could be seen and chromosomal examination revealed that only HeLa cells were present. Even when potoroo cells were first allowed

to cover 7/8 of the vessel before the HeLa cells were introduced, after three subcultures, when the cells were kept at 37°C, the potoroo cells disappeared from the population. It seemed as though potoroo cells could not stand the more anoxic conditions imposed on them by the HeLa cells settling on top and finally covering them completely.

Mixed cultures were set up afresh with even numbers of HeLa and HPK1 cells. After 12 to 18 hours at 37°C to allow for a quick attachment of both types of cells to the vessel, the cultures were placed into an incubator at 27°C. After two weeks at this lower temperature, many of the HeLa cells died and floated off the bottom, whereas the potoroo cells established themselves firmly and were more numerous than the former. On restoring the temperature to 37°C after change of medium, the HeLa cells multiplied more rapidly and after 5 days there were even numbers of both types of cells. At this stage, the cells were subcultured into 3 new vessels and the temperature was alternated between 37°C and 27°C in an effort to keep the numbers of both cell types fairly even. If cultures were kept at room temperature for 1 day (22 to 24°C), the HeLa population suffered greatly and the potoroo cells gained the upperhand. So that this provided a quick method of evening up numbers if the HeLa cells had been allowed to overcrowd the HPK1 cells.

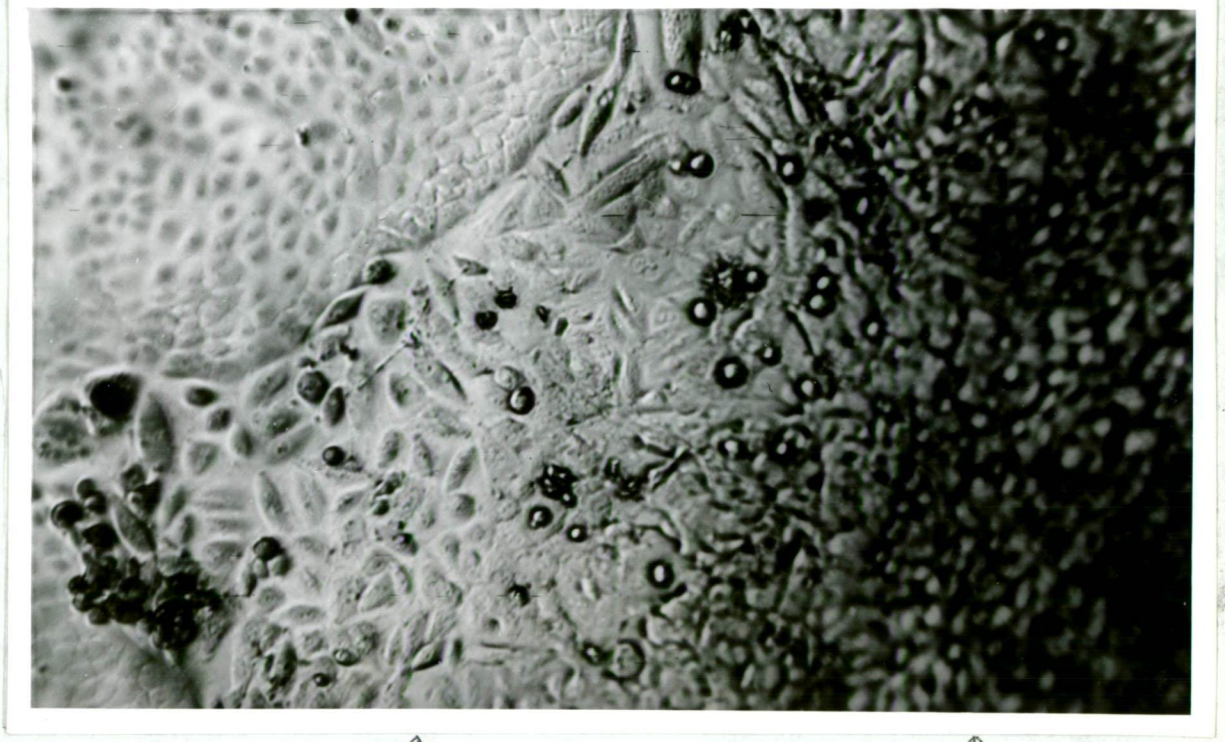
After 15 passages, i.e. 3 months from the initiation of the mixed cultures, versene treatment for 10 minutes (which usually removed adequately the HPK1 and HeLa cells) left quite a number of cells attached to the bottom of the vessel. After subculturing the cells that had come off, the remaining cells were covered with 5 ml of complete medium and maintained

at 37°C. After 5 days three populations of cells could be distinguished growing well at 37°C: HeLa cells, potoroo kidney epithelial cells (HPK1), and a new type of cell, much larger than the others, spindle shaped with clear cytoplasm like HPK1 (Photos 5, 6 and 7).

Soon after the appearance of this new cell type, I had to attend a conference in another state, and in the absence of research assistance it was decided to leave the mixed culture at room temperature for the four days, in the hope that the cells would be thus maintained in a fairly static condition and in particular that the HeLa cells would not overcrowd the other cell types. It was intended to try to isolate the new cell type by short treatment with versene or with trypsin in the hope that one or other cell type could be preferentially removed. However, after a few days at room temperature it was found that most of the spindle shaped cells had floated off the bottom of the vessel. The remainder of the culture (HPK HeLa 1) was subcultured and has been maintained to the present day, i.e. for $2\frac{1}{2}$ years, but it is completely potoroo epithelial, with contact inhibition, clear cytoplasm and at the last analysis HeLa chromosomes were completely absent. Apparently the four days at 22° to 24°C not only eliminated the new spindle shaped cells, but the cooler conditions favoured the potoroo cells so much that the HeLa cells disappeared completely from the population.

It is possible that the large spindle shaped cells that appeared in the mixed cultures were not hybrid cells but HPK1 cells enlarged by endoreduplication through being kept at lower temperatures. However, HPK1 cells have been kept at 27°C for up to 20 months with no sign of large spindle-

Photo 5

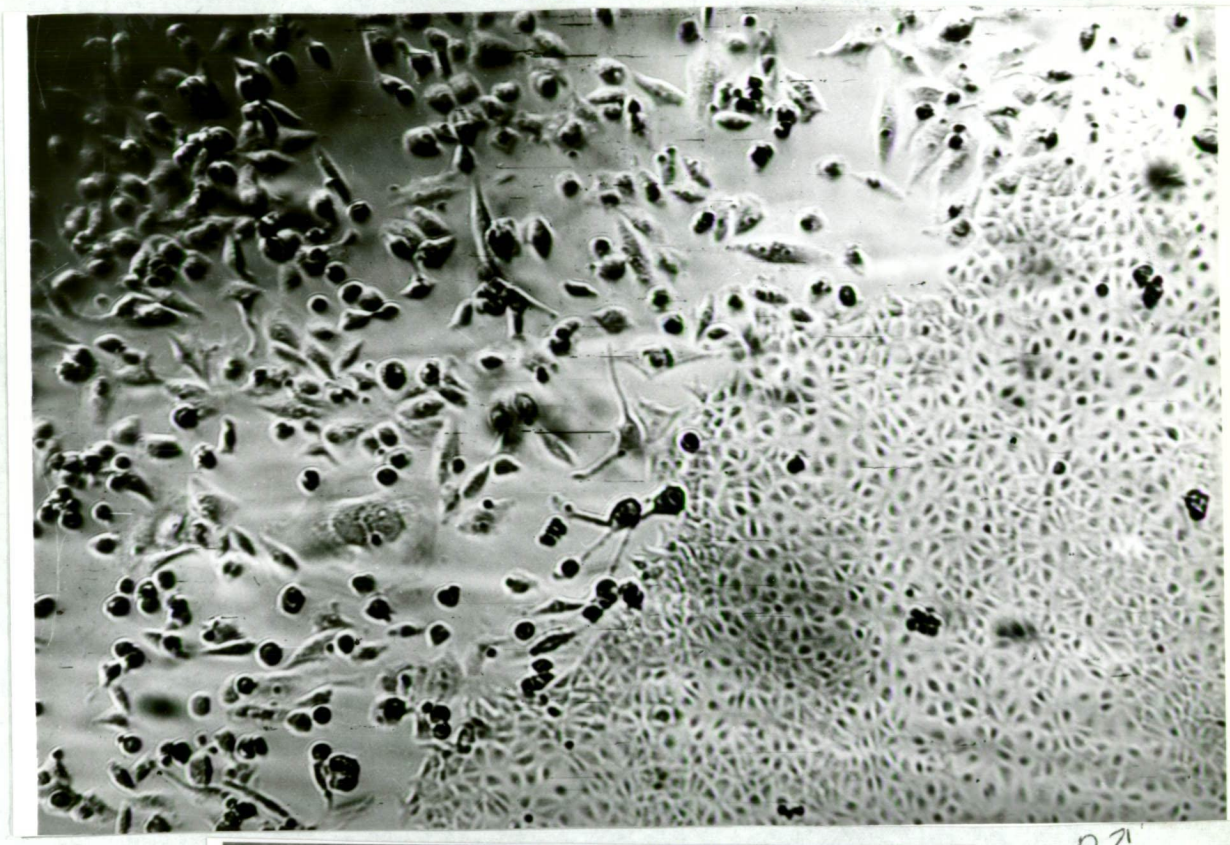


A. HPK1 cells - potoroo epithelial cells, with contact inhibition.

B. New cell type, larger fusiform cells.

C. HeLa cells without contact inhibition already more than one layer thick, some of the rounded HeLa cells can be seen on top of the spindle-shaped cells (B).

Photo 6



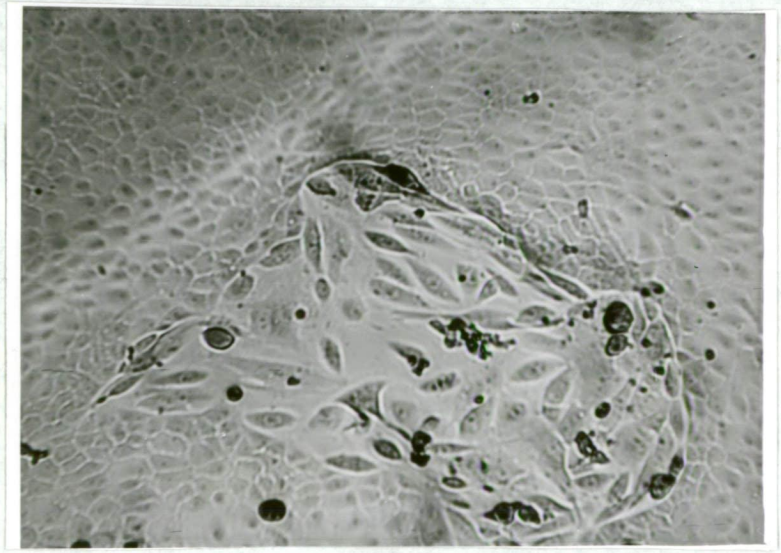


Photo 7 A group of the larger, spindle shaped cells surrounded by the potoroo epithelial cells.

shaped cells of this type. Potoroo testis fibroblasts HPT1 cells have produced odd shaped cells and some giant cells when kept at room temperature or 27°C for over 6 months; but these large cells always disappeared from the population when the temperature was restored to 37°C. The new spindle-shaped cells that appeared in our mixed cultures on the other hand grew well and kept pace with the HeLa and HPK1 cells at 37°C, but presumably could not tolerate the lower temperatures and in a few days became non-viable. It would be of interest to repeat the experiment, and in the light of experience in balancing the potoroo and HeLa cell populations by adjustment of temperature it may be possible to obtain this new cell type. This could be isolated by cloning or differential removal of cells with chelating agents. A study of its karyotype would be of considerable interest.

V Irradiation of Long-term Cultures

It has been known for some time that differences in chromosomal aberrations occur at metaphase according to the stage of the cell life cycle when the Xray dose was delivered[31]. For cells in G_1 (pre-DNA synthesis) the chromosome exhibits constant sensitivity to radiation[32]; as the cells pass towards metaphase, their sensitivity to Xray damage increases, but close to metaphase this sensitivity drops again[33]. At the onset of S (the stage of DNA synthesis) the sensitivity of the chromosome to Xrays doubles compared with the sensitivity observed in G_1; a further slight increase occurs through S up to G_2 (post-DNA synthesis), when another more definite increase in sensitivity is observed.

Wolff[34] postulated that the apparent difference between the pre-split G_1 chromosomes and post-split S and G_2 chromosomes could be due (1) to a bias in scoring procedure and (2) to the formation of a new type of aberration (the iso-chromatid deletion) in post-split chromosomes which is not present in chromosomes irradiated in G_1. He pointed out that in post-split chromosomes it is possible to score both symmetrical and asymmetrical interchanges, whereas only asymmetrical interchanges (dicentrics) and intrachanges (rings) can be scored in the pre-split chromosomes. Evans, Savage and Scott[35,36] suggested that this apparent gradual increase in sensitivity is probably caused by an increase in the number of double chromosomes produced as S progresses and not by the actual increase in the sensitivity of the chromosome material during this period. The experiments on which the above findings are based were carried out with plant material, mostly Vicia faba.

Brewen[37] (working with Vicia faba) found that for cells going from S to G_2, the number of aberrations increased by a factor greater than 2. This increase he found occurred only in two-hit aberrations, while the frequency of one-break aberrations remained constant from S through G_2. He concluded that this increase is a result of an increase in the number of sites within which an exchange can occur and not an increase in the sensitivity of the chromosome strands to Xrays. In very late G_2 or early prophase there was a sudden decrease in the number of observable aberrations. Brewen interpreted this as due to a decrease in the number of sites and also due to a possible decrease in the sensitivity of the chromosome strand since both one-break and two-break aberrations decreased

in frequency.

Hsu, Dewey and Humphrey[38], working on Chinese hamster cells found that, in parallel with results for plant material, cells in G_2 were 3 to 4 times more radiosensitive than those in S. They also found that the chromosome acts as a double structure to radiation when the irradiation is given at the commencement of DNA synthesis. It has also been found that HeLa S3 cells (human carcinoma) became increasingly sensitive as they progressed through G_2[39].

Using colony survival in the Chinese hamster cells in vitro, Morton and Sinclair[40] found that the most sensitive periods were G_2 and M (mitosis) followed in order of decreasing sensitivity by G_1, early S and finally late S.

Another difference is that breaks remain open (i.e. capable of rejoining) for different lengths of time in different parts of the cell cycle. When chromosome breaks are produced in a cell, these primary breaks may (1) reunite normally, resulting in no visible aberrations (2) fail to heal and give rise to one-hit aberrations or (3) reunite with another break to produce a two-hit aberration. The frequency of all types of chromosomal breaks scorable at metaphase is dependent on three factors:

(1) the frequency of primary breaks

(2) the damage done to the repair system and

(3) the proximity of broken ends at time of irradiation. The length of time the breaks remain open would depend on (2), i.e. the damage done to the rejoining system.

It is generally considered that the number of breaks observed at the

time of scoring is but a small fraction of the number of primary breaks resulting at the time of irradiation and that with a few exceptions, the majority of breaks are repaired shortly after the dose has been delivered[41].

Sax[42] working with Tradescantia showed that continuous radiation produces more damage than does intermittent radiation with respect to two-break types of aberrations. A number of independent investigations on Tradescantia[43,44] showed that the frequency of two-hit aberrations is reduced if the Xray dose is fractionated and that this reduction of the two-hit events is due to the restitution of breaks during the time interval between fractionated exposures. If two doses are given so close in time that all the breaks induced by the first dose are still open, then the yield of two-break aberrations will be proportional to the square of the total dose administered. If, however, the time between the two doses is long enough for repair to have occurred, then the yield of the two-hit aberrations will simply be the sum of those induced by the two separate doses.

Much work has been done on restitutional repair in plant chromosomes[45-49], the time for which varies from 5 minutes to 4 hours. Dewey and Humphrey[50] showed when hamster cells are irradiated in G_1, radiation-induced breaks remained open (i.e. capable of forming exchanges) for less than 5 minutes, whereas, if the cells were irradiated in S, chromosomal breaks remained open for at least an hour.

As little has been done in this field on mammalian cells, irradiation effects in Potoroo cell cultures were studied. Potoroo has a low chromosome number, almost half the number of chromosomes of the Chinese hamster.

The HPK1 line was used in these experiments because of its proven stability in continuous culture. It was imperative to be able to label the cells with tritiated thymidine (HTdR) so that the stage of the cell cycle could be ascertained by autoradiography at the time each metaphase was scored for damage.

In the absence of autoradiographic facilities, the project was planned as a joint one with Mr. Keith Brown, Australian Atomic Research Establishment. The HPTK1 cells were sent to Mr. Brown at Lucas Heights. Cultures grown at 37°C were treated with tritiated thymidine (HTdR), irradiated, slides were made, stained and sent to Hobart for scoring. They were then sent back to Lucas Heights for their development and on their return to Hobart, it was possible to ascertain by examination for silver grains at what stage each scorable metaphase was during irradiation.

Experimental

HPK1 cells exhibiting 96% diploidy were cultured in 199 medium supplemented with 8% foetal calf serum. Falcon plastic 30 ml flasks were seeded with 0.55 to 0.75 x 10^6 cells in 4 ml medium and incubated 48 hours at 37°C with a medium change after 24 hours. After 48 hours, the conditioned medium was sucked off and saved. The cells were pulse labelled for 10 minutes at 37°C by adding 4 ml fresh medium containing 1μCi/ml of HTdR (5.0 Ci/mM). The labelling was terminated by sucking off the medium and washing the cells twice with 4 ml of fresh warm medium containing 10μg/ml unlabelled TdR. Finally the pooled conditioned medium, to which 10μg/ml of unlabelled thymidine had been added, was returned to the flasks.

Immediately afterwards the flasks were irradiated at room temperature employing a Philips constant potential Xray machine operated at 250 KV and 15 m.a. and filtered by 0.5 mm Cu, 1.0 mm Al. The dose rate was 100r/minute. Treatments were given as outlined in Table IV.

Table IV

Between the 2 doses of irradiation, cells were incubated at 37°C.

A1	100 roentgens	-	30' wait	-	100r
A2	100 r	-	10'	-	100r
A3	100r				Slides were made 4 hours post irradiation
A4	200r				
A5	control - no irradiation				
B1	100r	-	30'	-	100r
B2	100r	-	10'	-	100r
B3	100r				Slides were made 12 hours post irradiation
B4	200r				
B5	control - no irradiation				

The temperature was then restored to 37°C. Colchicine at a concentration of 0.6 µg/ml was added to series A and B for 2 hours before the termination of cultures. After 4 and 12 hours respectively, the medium was removed and replaced by warm (37°C) versene. After 6 to 10 minutes at 37°C each cell suspension was centrifuged (100g for 6 minutes), the supernatant was removed and replaced with 1 ml of B.S.S. The cell suspension was evenly dispersed by pipetting, then 2 ml of warm distilled

water was added and the cells were mixed gently in this hypotonic solution which was then placed in a warm water bath (37°C) for 10 minutes. Fixative was added (3 ml of 1:3, glacial acetic acid:absolute ethanol) and the cell suspension was left at room temperature for 30 minutes, then centrifuged. The supernatant was removed and replaced with 2 ml of fresh fixative, in which the cells were well dispersed and left for a further 30 minutes at room temperature. After this time, the cell suspension was centrifuged, the supernatant removed and replaced by 1 ml of 60% acetic acid and the cells again dispersed by gentle pipetting. After five minutes, slides were made by dropping a few drops of the cell suspension onto a clean slide, then drying the slides at 60°C. After being stained with Leishman's the slides were scored.

Results

Except for the control A5, the cells gathered 4 hours after irradiation showed few mitoses and significant results were not obtained. Cells (B) gathered 12 hours after irradiation showed an adequate mitotic index. The results of the scoring of these cells are given in Table V.

Unfortunately the labelling of these slides proved faulty possibly because of the length of time which had elapsed between making the slides and their final development. It was thus not possible to ascertain at what stage each metaphase was at the time of irradiation. Further experiments on HPK1 cells using HTdR showed that after a dose of 200r at 100r/minute, 80% of the cells were in S phase at the time of irradiation when the cells were harvested 12 hours after the irradiation dose was delivered.

Table V

	Chromatid deletions	NUpd	SU	NUp	NUd	Total Isochromatid deletions	TRANSLOCATIONS Chromatid	TRANSLOCATIONS Chromosomes	No. of Metaphases scored
B1, 100r-30'-100r	12	30	9	14	5	58	3	0	100
				28					
B2, 100r-10'-100r	13	16	7	7	9	39	2	0	100
				23					
B3, 100r	13	7	11	15	3	36	2	0	100
				29					
B4, 200r	9	30	10	15	7	62	2	0	100
				32					
B5, control	7	10	1	0	1	12	0	0	100
				2					

Scores for B1, B2, B3 and B4 have been corrected for spontaneous aberrations by deducting control (B5) figures.

Scoring was done only on diploid cells which averaged out to 95% on B slides.

SU = complete sister-strand reunion

NUp = non-union proximal (i.e. centric portion)

NUd = non-union distal (i.e. acentric pair)

NUpd = non-union both proximally and distally

This indicated that the majority of cells scored in the B series were in S at the time of irradiation.

From Table V it can be seen that there is virtually no chromosome damage so that it can be assumed that the majority of cells scored were in S or G_2 at time of irradiation. The fact that there are only a few chromatid translocations indicates that larger doses and higher dose rates will have to be used in future experiments to ascertain the time in S during which the broken chromosome ends remain open. It is of interest that the greatest damage appears as iso-chromatid breaks. Wolff[51] working with plant material found that the first iso-chromatid deletions always appear in samples of cells containing chromatid and not chromosome aberrations, i.e. they are induced in S and G_2 cells. He also found that they undergo some sister-strand reunion - SU, NUp or NUd. Chromosome terminal deletions from G_1 cells never undergo rejoining and always appear as NUpd. From the fact that the HPK1 cells have a generation time of $22\frac{1}{2}$ hours and from data which showed that at least 80% of the cells were in DNA synthesis when irradiated, it is most unlikely that any of the cells were in G_1 when the Xray dose was delivered. Thus in animal cells a large percentage of cells evidently show isochromatid breaks of the NUpd type. This fits in with the observations of J.G. Brewen[52] who noticed the same phenomenon when working with Hamster cells.

Chromatid breaks per 100 cells were obtained by adding the numbers of chromatid deletions, iso-chromatid breaks and double the number of chromatid exchanges (Table VI). For Chinese hamster cells the iso-chromatid deletions were found to exhibit linear kinetics and to be proportional to

the dose[53].

Table VI

	Breaks %	Damage/cell/r	Cells counted	M.I.*
B1 100r-30'-100r	76	.0038	100	1.0
B2 100r-10'-100r	56	.0029	100	1.1
B3 100r	53	.0053	100	1.5
B4 200r	75	.0037	100	1.3
B5 control	14	-	100	2.0

* M.I.s were calculated by counting 10,000 cells for each of the cultures

Damage produced by 100r is 53% and by 200r is 75%. The slightly lower value obtained for 200r can be ascribed to the fall in the mitotic index (by 14%) which probably resulted from some of the more damaged cells at the higher dose not reaching metaphase 12 hours after irradiation. Seeing that most of the damage is of the one-event type, i.e. chromatid and iso-chromatid deletions, one would expect that fractionating the dose would not change the damage scored and the results support this: for 200r given as one dose the damage is 75% while 200r delivered in two doses of 100r separated by a 30 minute interval produced 76% damage.

The very low damage scored for B2, 100r-10'-100r, which is almost identical with the damage produced by a single dose of 100r is difficult to understand and will require confirmation; the whole experiment will be repeated and extended. Humphrey and Dervey[50] found for Chinese hamster cells a differential radiation-induced mitotic lag depending on the length

of the time interval between two doses. They found that for cells pulse labelled with HTdR and irradiated with a single dose of 300 rads, or two equal doses each of 300 rads, separated by intervals varying from 10 to 60 minutes, 46% of the cells that were scored were labelled, i.e. 46% had been irradiated in S. However, after a dose of 600 rads delivered over 1 minute, the percentage of cells in S rose to 78%. It is possible that in the present experiments delivery of a dose of 200r over 10 minutes inhibited the cells in G_2 to such an extent that hardly any reached metaphase after 12 hours. It has been shown that cells in G_2 are most sensitive to radiation damage, and this may account for the low damage scored for B2. The mitotic indices for B1 (100r-30'-100r) and B2 (100r-10'-100r) were found to be lower by 23% and 15% respectively than for B4, i.e. when 200r was delivered as one dose.

In the above experiments the average number of breaks/cell/roentgen is .0039 as compared to Bender and Gooch's[54] value of .0031 breaks/cell/r for human leucocytes in culture. In their experiments, the cells in a 66-hour-old culture were irradiated with doses of 25r and 50r and the chromosome aberrations were scored 6 hours later. Considering that in their experiments the time between fixation and irradiation was 6 hours compared with 12 hours in our experiments, it is possible that some of the more damaged cells did not reach metaphase at the time of scoring. Brewen[55] found that when the corneal epithelium of the Chinese hamster is irradiated with Xrays at three different dose rates, the observed frequency of chromatid aberrations, both chromatid deletions as well as chromatid exchanges, is different at each of the dose rates employed.

The lower dose rates used by Bender and Gooch[54] could also account for the fact that their final damage is somewhat lower than ours. The mean damage obtained in our previous experiments on irradiation of potoroo leucocytes at 100r, 200r and 300r at 25r/minute in G_1 was .0033 hits/cell/r.

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Appendix

Published Papers

1. Bick, Y.A.E. and Jackson, W.D., A Mammalian X-O sex-chromosome system in the Monotreme Tachyglossus aculeatus determined from Leucocyte Cultures and Testicular Preparations, Amer. Natur., 101, 79-86, 1967.
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A MAMMALIAN X-O SEX-CHROMOSOME SYSTEM IN THE MONOTREME *TACHYGLOSSUS ACULEATUS* DETERMINED FROM LEUCOCYTE CULTURES AND TESTICULAR PREPARATIONS

Y. A. E. BICK AND W. D. JACKSON

Departments of Zoology and Botany, University of Tasmania

Recent advances in cytological technique and evaluation of the DNA content have stimulated interest in the basic cytological relationships within the animal kingdom. In particular, evidence suggests that although the chromosome number and size varies within rather wide limits in eutherian mammals, the DNA content is relatively constant (Allfrey, Mirsky, and Stern, 1955). Estimates of the chromosome volume by area determinations in colchicined metaphases have been made in various animal groups (Ohno, Beçak, and Beçak, 1964; Ohno et al., 1964; Beçak et al., 1964). These results show that the birds have a total chromosome area slightly less than half that of mammals, in agreement with data from DNA measurements. The serpents appear to be similar to the birds in DNA content and chromosome volume, a similarity which is also strengthened by the well-known similarity in karyotypes composed of macro and micro elements. Marsupials are characterized by large chromosomes and small numbers but the DNA content and volume agree with that of eutherian mammals.

It would be of interest in light of the above knowledge to establish the relative position of the monotremes. The karyotype of the Platypus has been investigated by Matthey (1949) although with the techniques available at the time the number (70 ± 10) could not be ascertained with accuracy. Van Brink (1959) has published a report of the karyotype in Echidna giving numbers for the male between 62–64. Matthey (1949) remarks that the karyotype in these monotremes is morphologically similar to that in the birds and Chelonian reptiles.

In most mammalian species studied there is a typical XX-XY sex chromosome mechanism, and the male is the heterogametic sex. However, there is a $XX - XY_1Y_2$ mechanism in two macropod marsupials, *Potorous tridactylus* and *Wallabia bicolor* (Sharman and Barber, 1952) and in at least one eutherian mammal, *Sorex araneus* (Bovey, 1949). The sex-determining system in *Monotremata* is completely unknown.

In this work, an attempt has been made to resolve the sex-determining system of *Tachyglossus aculeatus* (the Echidna or spiny anteater) and to determine the karyotype. This work is preliminary to the investigation of DNA content and chromosome area in both monotremes. Metaphases from colchicine treated short-term cultures of leucocytes from the peripheral blood of the male and female Echidna gave suitable preparations from

which the chromosome number could be determined without ambiguity. In addition, chromosome spreads from the male Echidna were obtained by subjecting testis material to trypsinization and hypotonic treatment for examination of mitosis and meiosis in spermatogenesis.

MATERIALS AND METHODS

Chromosome spreads from testis

The testes of a mature Echidna were removed, chopped finely, and then subjected to trypsinization (0.25% trypsin solution) with agitation by a magnetic stirrer for 20 min. The suspension of cells was centrifuged at 150 g for 7 min. The supernatant was removed and the cells were resuspended in balanced saline solution; clumps were broken up by gentle pipetting. This suspension was then placed into fresh tubes (1/2 ml in each) and 1½ ml of warm (37 C) distilled water added to each tube. After thorough pipetting to ensure penetration of the hypotonic solution and to prevent any clumps of cells forming, the tubes were immersed in a water bath at 37 C for 12 min. After this time, 1½ ml of glacial acetic acid and absolute ethanol fixative (1 : 3) were added to each tube, and the cell suspension was again gently pipetted. The tubes were allowed to stand at room temperature for half an hour. They were then centrifuged, the supernatant was removed, and replaced by 1½ ml of freshly prepared fixative. After 30 min, slides were made by pipetting a couple of drops onto a clean slide and adding 4 to 5 drops of 50% acetic acid. The slides were dried either by gentle heating over a bunsen flame or on a warm plate (50 C). Slides were rinsed in absolute methanol, air dried, and stained for 10 min with Leishman's stain.

Leucocyte culture

The leucocytes were cultured by a modified method of Moorhead et al. (1960). Medium 199 (Parkers) with 10% foetal calf serum was used (both obtained from the Australian Commonwealth Serum Laboratories). The phytohaemagglutinin (Wellcome) was dissolved in complete medium. The final concentration of phytohaemagglutinin (PHA) was 5½% (1 mg/ml).

Blood was removed from the heart with a heparinized sterile syringe and placed in 5 ml aliquots into tubes containing a couple of drops of heparin and PHA. After 10 min the blood was gently centrifuged at 100 g for about 5 min until about 40% of leucocyte suspension in plasma appeared on top. This upper layer was removed into fresh tubes and centrifuged for 10 min at 150 g when all the leucocytes formed a pellet at the bottom. All the supernatant was removed and the cells resuspended in 4 ml of complete medium. After centrifuging for 10 min, the supernatant was again removed and 1 ml of complete medium was added to each tube. Half of this suspension was added to each culture bottle which contained 8½ ml of complete medium.

Cultures were incubated at 27 C, 33 C, and 37 C. Colcemid (Ciba) was added for 2, 48, or 58 hours of the final culture period. The final colcemid

concentration was either 0.007 % or 0.01 %. Cultures were stopped on the 3rd, 4th, 5th, 6th, 7th, 8th, and 9th day.

Unlike mammalian or marsupial leucocytes, those of the Echidna tend to adhere very firmly to the bottom of the glass vessels, and in some cases versene had to be used to free the cells. The leucocytes remaining in suspension tend to form clumps, so that vigorous pipetting is essential before hypotonic treatment can be applied. After the leucocytes were separated from the culture medium by centrifuging, the supernatant was removed except for 1/2 ml in which the cells were resuspended. The cells were then given hypotonic treatment, fixed, and slides were made in the way described above for testis cells.

RESULTS AND DISCUSSION

The physiological temperature range of the Echidna lies somewhere between 25 C and 30 C. Above 30 C its temperature rises with the environment. In the absence of efficient sweat glands, panting mechanism, and heat loss by vaso-dilation of surface capillaries, the animal dies of heat-stroke at 37 C if unable to burrow. The Echidna caught in winter by us had a temperature of 29 C whereas the one found in summer had a cloacal temperature as high as 33 C.

The results of the temperature tests for leucocyte cultures are given in Table 1. The concentration of colcemid used is much higher than that usually employed for leucocyte cultures of eutherian mammals and approaches the concentrations that have been used successfully for amphibian cells (Seto, Pomerat, and Kezer, 1964). The best results were obtained with a final colcemid concentration of 0.01 %. The concentration of PHA is also higher than that used for mammalian leucocytes and approaches those that were successfully used to culture amphibian leucocytes.

Cultures kept at 37 C showed no divisions after 72 hours; after 5 days at this temperature, the nuclei remained small, and a number of them became

TABLE 1

Temp.	Colcemid treatment (hours)	Culture time (days)	Mitotic index (metaphases/1,000 cells)
27 C	2	3	none
27 C	2	6	none
27 C	48	6	mainly prophases
27 C	58	6	1-2
27 C	48	7	5-20
27 C	48	7	> 20*
27 C	48	8	5-20
27 C	48	9	5-20
35 C	48	5	1-2
37 C	24	3	none
37 C	48	5	none

*Final concentration of colcemid was 0.01 % in this treatment compared with 0.007 % in all other series.

pycnotic. The best results were obtained from cultures kept at 27 C. Leucocyte cultures of eutherian mammals grown at 37 C show a mitotic peak after 72 hours. At a temperature 10 degrees lower, one would expect, because of the lowered metabolism, that the onset of the mitotic wave would be delayed. So it is not surprising that for Echidna leucocytes the highest mitotic index was obtained around the 7th day of culture. However, there appears to be no sharp peak in the number of mitoses. Forty-eight hours of colcemid treatment is necessary to obtain a reasonable mitotic index, and a high level of divisions is maintained for the following 48 hours.

The chromosome number in the two male Echidnas examined was found to be 63 and in the single female 64. At least 20 metaphases which gave these numbers were scored for each sex.

Metaphases obtained from leucocyte cultures gave many suitable spreads (Fig. 3). Drawings from projected photographic negatives are shown in Fig. 1 and 2. With the long colchicine treatment, the chromosomes become contracted and the chromatid arms are well separated at the time of fixation. The position of the centromeres could thus be determined with accuracy.

From Fig. 4 and 5 it can be seen that the largest chromosomes are acrocentric. The pair in position 5 possesses two satellites separated from the centromere by a long heterochromatic segment. This pair of chromosomes is very easily recognized, although sometimes the heterochromatic region may be relatively condensed and shortened. The pair in number 8 position has a similar pair of satellites; but the heterochromatic region is shorter relative to the other arm of the chromosome, and the satellite bodies are not as distinct as in the former pair.

FIG. 1. Chromosome complement of a male Echidna obtained from testis without any colchicine treatment.

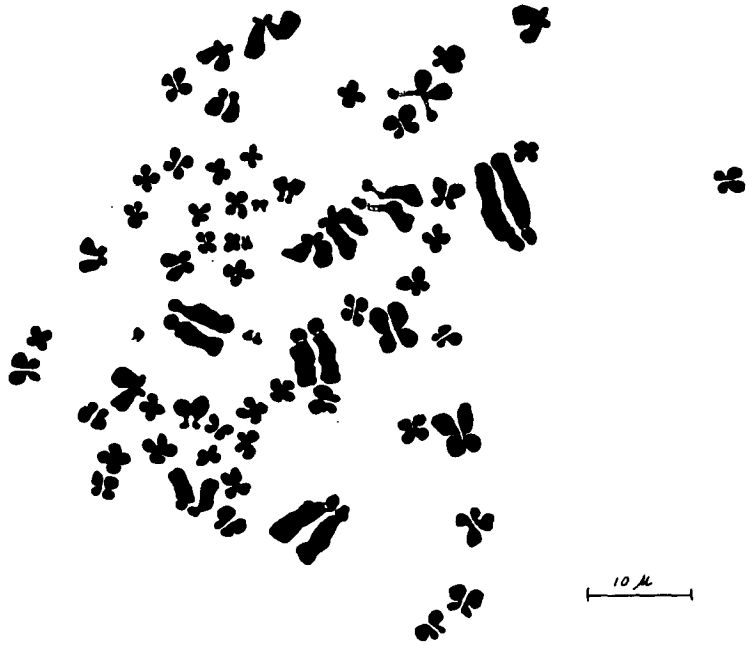

FIG. 2. Chromosome complement from leucocyte culture of female Echidna, after 48 hours colcemid treatment.

The chromosomes from pair number 9 to pair 26 are metacentric or submetacentric, gradually decreasing in size, often by so little that there is difficulty in pairing the homologues. The smallest pairs, 30 and 31, are much smaller than the rest of the chromosomal complement. The pair 31 is about half the size of the pair number 30. Both the smallest pairs appear to be telocentric or subtelocentric.

In the male Echidna there is one large metacentric chromosome which we have called the X chromosome. This is easy to recognize as it is the largest metacentric chromosome present. In the female, there are two of these chromosomes. (It would appear that the large acrocentric thought to be the sex chromosome by Van Brink [1959] is the largest autosome.) Thus the somatic numbers are 64 in the female, 63 in the male. This result strongly suggests a sex mechanism of XX in the female and XO in the male, the male of the Echidna being the heterogametic sex. These results would apparently present the first case of an XX – XO sex chromosome system in a mammal (White, 1960).

It is possible that the Echidna represents some form of mosaicism similar to that in the creeping vole (Ohno, 1963). The absolute confirmation of an X – O system from a study of meiosis is difficult because material in meiosis is only available during a restricted period, and the large chromosome number makes it difficult to determine the presence of a monovalent. However, from our examination of testis preparations, we are reasonably

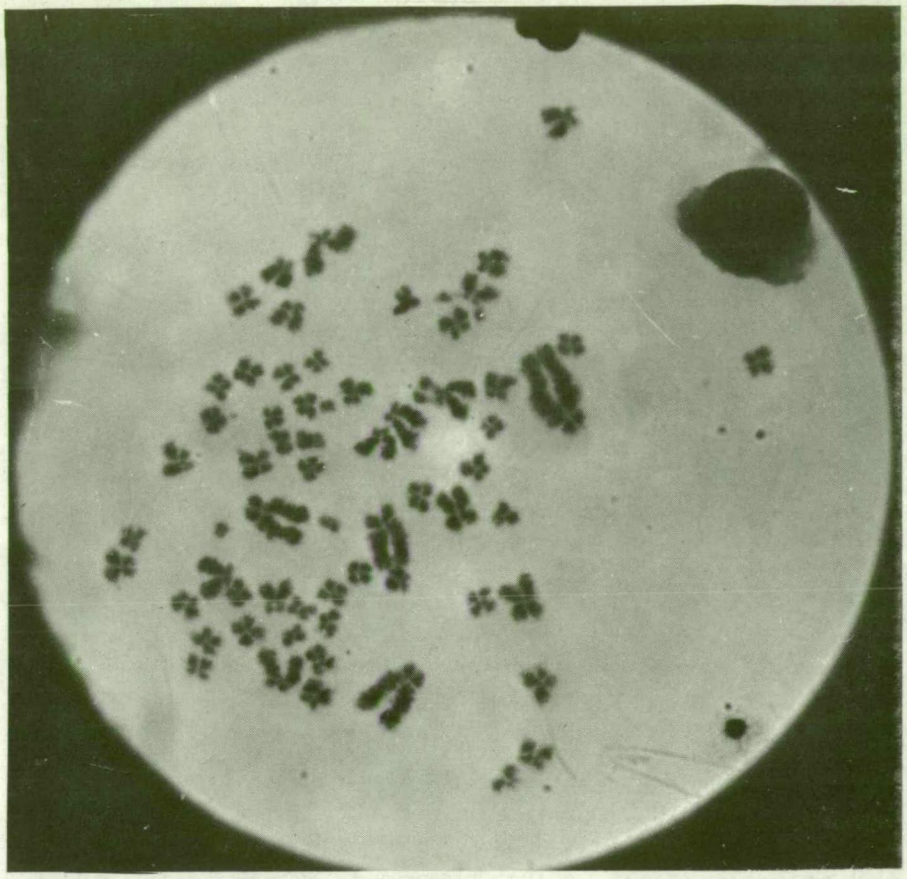

FIG. 3. Metaphase chromosomes from female Echidna obtained from leucocyte culture.

confident that the meiotic configuration shows 31 bivalents and one monovalent. Certainly, the mitotic divisions in the testes show the number 63 (Fig. 1) seemingly excluding mosaicism.

Both White (1945) and Matthey (1949) have noted a resemblance between the chromosome morphology of the monotremes and that of birds and reptiles. In leucocyte cultures, the chromosomes of Echidna show a marked range in size. However, there is a gradation, and even the smallest chromosomes are considerably larger than the microelements typical of the birds. All chromosomes have a well-defined centromeric constriction. It would appear from comparison with the work of Beçak et al. (1964) that the chromosome morphology in monotremes is to be regarded as an extreme mammalian type strongly resembling that found in the crocodiles and turtles.

We have begun similar studies on the Platypus in order to compare the chromosome morphology and sex chromosome mechanisms in these monotremes. These preliminary investigations indicate that the chromosome

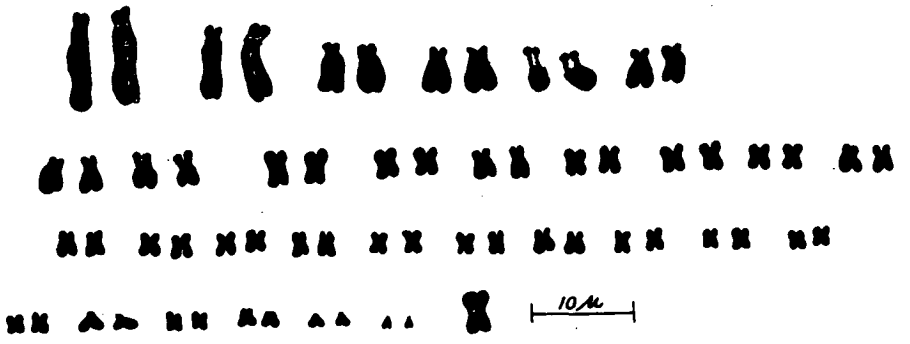

FIG. 4. Idiogram of male Echidna: 31 pairs of autosomes and one X chromosome.

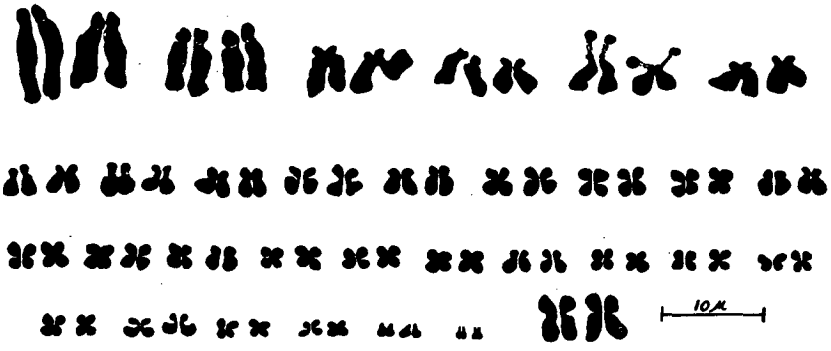

FIG. 5. Idiogram of female Echidna: 31 pairs of autosomes and two X chromosomes.

number of *Ornithorhynchus* is approximately ten less than in *Tachyglossus*. An X – O sex mechanism is also indicated.

SUMMARY

The chromosomal complement of the monotreme Echidna has been found to be 64 in the female and 63 in the male which is the heterogametic sex. This indicates an XX female, XO male sex-chromosome mechanism. Metaphase chromosome spreads were obtained from the testis and from leucocyte cultures. Details of conditions of culturing Echidna leucocytes from peripheral blood are given.

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